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UNIVERSITY OF  
**ANALYSIS OF PROTECTIVE CELLULAR IMMUNE  
RESPONSES AGAINST HEPATITIS C VIRUS**

**A. NASSER SEMMO**

**SUBMITTED FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY**

**2006**

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## Abstract

Hepatitis C virus (HCV) infection is a major cause of liver damage, with virus-induced end-stage disease such as liver cirrhosis and hepatocellular carcinoma resulting in a high rate of morbidity and mortality worldwide. There is now considerable evidence that CD4<sup>+</sup> T cell responses to HCV play an important role in the outcome of infection. However, the functional status of HCV-specific CD4<sup>+</sup>ve T cells in persistent infection is poorly understood and it may be necessary to use a variety of techniques in their detection and analysis.

The aim of my thesis is to determine aspects of cellular immunity that are associated with viral control, here mainly focusing on CD4<sup>+</sup> T cell responses.

The first data chapter (chapter 3) gives an analysis of RIBA-indeterminate blood donors negative for HCV-PCR in whom HCV-specific T cell responses were identified, typically focused on core-derived peptides, suggesting previous exposure to HCV.

I next analyse the cytokine secretion patterns in chronically HCV-infected patients and compare them with those with resolved infection (chapter 4). Using overlapping peptides, I have been able to identify HCV-specific CD4<sup>+</sup> T cells in persistent infection that recognise the mainly conserved core region. These cells characteristically produce IFN- $\gamma$  but not IL-2 (IFN- $\gamma$ <sup>+</sup>/IL-2<sup>-</sup>). In the next chapter (chapter 5) I show that these cells have lost their ability to proliferate (IFN- $\gamma$ <sup>+</sup>/IL-2<sup>-</sup>/proliferation<sup>lo</sup>).

In the final data chapter (chapter 6), I studied the T cell responses in a cohort of seven individuals with antibody-deficiency (CVID), who received early interferon therapy after HCV infection through contaminated  $\gamma$ -globulin. Even in the absence of antibody responses, substantial HCV-specific T cell responses could be recovered.

The implications of my findings are discussed at the end of each data chapter. A general discussion of the overall findings and future work that may evolve from this work is outlined in chapter 7. Here, I also give some preliminary data on a cohort of acutely HCV-infected individuals with and without HIV infection, showing that HCV-specific CD4<sup>+</sup> T cell responses in acute HCV are significantly reduced in co-infection when compared with HCV mono-infection.

Overall the data in this thesis provide novel insights into the immune status in specific patient groups, and the functional status of HCV-specific CD4<sup>+</sup> T cells in persistent infection.

## **Collaborations**

Chapter 3 “ T-cell responses and previous exposure to hepatitis C virus in indeterminate blood donors” was a collaborative effort between our group and Neil Smith, Craig Taylor and John Kurtz at the National Blood Service in Birmingham, UK.

In “Other studies performed” (Chapter 7): “Reduced HCV Specific CD4<sup>+</sup> T Cell Responses in HIV/Acutely HCV Co-Infected Individuals” was performed in collaboration with Mark Danta, Dave Brown and Geoff Dusheiko from the Royal Free Hospital in London and Paolo Fabris from the S. Bortolo Hospital in Vicenza/Italy.

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## ABBREVIATIONS

aa	amino acid
Ab	antibody
ALT	alanine amino transferase
APC	antigen-presenting cell
B-LCL	B lymphocyte cell lines
C	core protein
CFSE	carboxy-fluorescein (diacetate) succinimidyl ester
cpm	counts per minute
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cells
DMSO	dimethylsulphoxide
EDTA	ethylene diaminetetracetic acid
dNTP	deoxynucleotide triphosphate
ds	double-stranded
E1	envelope 1
E2	envelope 2
EBV	Epstein Barr virus
eIF2- $\alpha$	eukaryotic initiation factor 2
ELISA	enzyme-linked immunosorbant assay
ELISpot	enzyme-linked immunospot
FACS	fluorescence-activated cell sorter
Fas-L	Fas Ligand
FCS	foetal calf serum
FITC	fluorescein isothiocyanate

Foxp3	forkhead protein 3 (transcription factor)
g	gram
H <sub>2</sub> O	water
HAART	highly active antiretroviral therapy
HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HVR	hypervariable region
ICS	intracellular cytokine stain
IFN- $\alpha$	interferon-alpha
IFN- $\gamma$	interferon-gamma
Ig	immunoglobulin
IL	interleukin
IRES	internal ribosomal entry site
ISG	interferon-stimulated gene
IU	international units
JAK-STAT	janus tyrosine kinase-signal transducer and activator of transcription
l	litre
LCMV	lymphocytic choriomenigitis virus
LDL	low density lipoprotein
m	milli
M	molar
mAb	monoclonal antibody
MHC	major histocompatibility complex

mins	minute
MU	million units
NANBH	non-A, non-B hepatitis
NK	natural killer
NK	natural killer (cells)
NS	non-structural
OAS	oligoadenylate synthetase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PEG	polyethylene glycol
PerCP	peridin-chlorophyll protein
PKR	protein kinase RNA dependent
RNA	ribonucleic acid
RIBA	Recombinant immunoblot assay
RT	room temperature
(RT) PCR	(reverse transcription) polymerase chain reaction
SFC	spot forming cell
SI	stimulation index
SIV	simian immunodeficiency virus
SVR	sustained virological response
TCR	T cell receptor
TLR	toll-like receptor
TNF	tumour necrosis factor
UTR	untranslated region

# CHAPTER 1

## INTRODUCTION

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## **1. Essential HCV background information**

### **1.1. Epidemiology**

The World Health Organisation estimates that 170 million people worldwide are currently infected with HCV. The prevalence of HCV seropositivity is estimated to be approximately 3% overall, and 0.7-1% in the United Kingdom. The majority of people (approximately 75%) who are seropositive will be viraemic (Seeff, 1997). Globally, HCV is five times more prevalent than Human Immunodeficiency Virus (HIV).

A significant number of infected individuals have acquired HCV through the transfusion of contaminated blood products before the introduction of HCV screening in 1990 (Alter et al., 1999b). For example, 90% of haemophiliacs who received factor-concentrates prior to 1985 are anti-HCV positive (Blanchette et al., 1994). The screening of donor blood products for anti-HCV antibodies and the testing of HCV RNA in pooled donor serum has now reduced the risk of infection to less than 1 in 103,000 transfused units (Schreiber et al., 1996). Injecting drug use remains an important risk factor for HCV, and other skin piercing procedures have been shown to transmit the virus (Hayes and Harkness, 2001). Sexual transmission has been described, but is an inefficient and infrequent route of infection (Wyld et al., 1997). Mother-to-infant transmission occurs in less than 5% of cases, but this rate is significantly higher in maternal co-infection with HIV (Ohto et al., 1994; Thomas et al., 1998). The precise mode of acquisition of HCV is uncertain in 20-30% of patients (Alter et al., 1992).

HCV-infected individuals are at risk of developing liver cirrhosis and hepatocellular carcinoma. Unlike hepatitis A virus (HAV) and hepatitis B virus (HBV) infection, there is no vaccine available to protect against HCV infection. Current treatment regimes are frequently traumatic for patients and therapeutically sub-optimal, particularly for genotype-1 infection. HCV infection is now a leading indication for liver transplantation in the United Kingdom and the U.S.A. Furthermore, HCV is now one of the main causes of morbidity and mortality in patients co-infected with HIV in developed countries (Mohsen et al., 2002).

Clearly then, a better understanding of the factors that mediate HCV viral control, whether in the context of spontaneous viral control or in response to therapy is needed so that new therapeutic regimes and vaccination strategies may be developed.

## **1.2. Molecular Virology**

Hepatitis C (HCV) is a positive-sense single-stranded RNA virus of the flaviviridae family of viruses. The HCV genome consists of 9600 nucleotides (9.6 kb), and contain two open untranslated regions (UTRs) and a single open reading frame that is 9000 nucleotides in length. Translation of the genome results in a polypeptide, 3000 amino acids (aa) in length that is post-translationally cleaved into ten structural and non-structural proteins (Figure 1.1). The polypeptide is flanked at each terminus by untranslated regions (UTR) that play an important role in viral replication. The 5' UTR contains an internal ribosome entry site (IRES) for the initiation of viral polyprotein translation, whereas the 3' UTR contains the signals which are necessary for the termination of viral replication.

The four structural proteins translated include core, E1, E2, and p7. HCV core, a highly basic protein, is thought to associate with HCV genomic RNA to form the viral nucleocapsid (Santolini et al., 1994). The two envelope proteins, E1 and E2, are post-translationally modified by Asn-linked glycosylation (Hijikata et al., 1991b; Matsuura et al., 1992). The E2 protein contains two hypervariable regions (HVR 1 and HVR 2) that are associated with a high mutation rate, which is thought to be the result of selection pressure by virus-specific antibodies (Hijikata et al., 1991a; Weiner et al., 1991). In addition, E2 contains the binding site for CD81, a member of the tetraspannin family that is expressed on a variety of cell types, including lymphocytes and hepatocytes, and is thought to be the HCV receptor or coreceptor for viral entry (Pileri et al., 1998). P7, an additional structural protein at the C-terminal end of E2, is a highly hydrophobic protein with ion channel activity (Griffin et al., 2004).

Six non-structural proteins have been identified in HCV, although a function has not yet been defined for every protein. NS2, NS3, and NS4a are thought to have protease activity, and the NS3 protein contains helicase activity in the C-terminal 500 amino acid residues (Fig. 1.1) (Grakoui et al., 1993; Kim et al., 1995). NS4b is a hydrophobic protein assumed to be membrane-bound, although its biochemical function is not currently known (De Francesco, 1999). NS5b encodes the RNA-dependent RNA polymerase (Lesburg et al., 2000). Although an interferon-sensitivity-determining region (ISDR) has been described in NS5a, the functional role of this protein is currently unclear (Enomoto et al., 1995; Enomoto et al., 1996).

### 1.3. Viral classification

HCV is the only member of the genus *Hepacivirus* and it has been classified within the *flaviviridae* family. Viruses belonging to this family (flaviviruses, pestiviruses, hepaciviruses, GBV-A, GBV-B and GBV-C /HGV) are all positive-sense single stranded RNA viruses. This classification is based upon the genomic organisation and sequence homology between these viruses (Choo et al., 1991; Robertson et al., 1998). Six distinct HCV genotypes and more than 50 sub-types have been identified on the basis of viral sequence homology (Bukh et al., 1995; Simmonds et al., 1994a; Simmonds et al., 1994b). The prevalence of genotypes differs by geographical location. The predominant genotypes in the U.K. and USA are 1a and 1b, followed by genotypes 2 and 3. Genotype 3 is particularly common in younger individuals with a history of intravenous drug abuse. Genotypes 4, 5 and 6 are common to Egypt, South Africa and South East Asia respectively. Genotype 2 is dominant in West Africa. On average over the complete genome, the six genotypes differ in 30–35% of nucleotide sites, with more variability concentrated in regions such as the E1 and E2 glycoproteins, whereas sequences of the core gene and some of the non-structural protein genes, such as NS3, are more conserved (Simmonds, 2004). For example, within the core protein, there is an 8% amino acid difference between the genotype 1a and 3a consensus sequences (16 aa out of 191 aa). As for genotype 1a and 1b the difference is about 1.6% (3 aa out of 191 aa). Genotype 2 is more distant from genotype 1 than is genotype 3. The lowest sequence variability between genotypes is found in the 5' UTR, where specific sequences and RNA secondary structures are required for replication and translation functions.

The RNA-dependent RNA polymerase encoded in NS5B lacks a “proof-reading function” which results in a very high HCV RNA mutation rate. This results in distinct but closely related multiple viral variants known as “quasi-species” within each infected individual (Alter et al., 1999a; Hoofnagle, 2002; Simmonds et al., 1994a; Simmonds et al., 1994b).

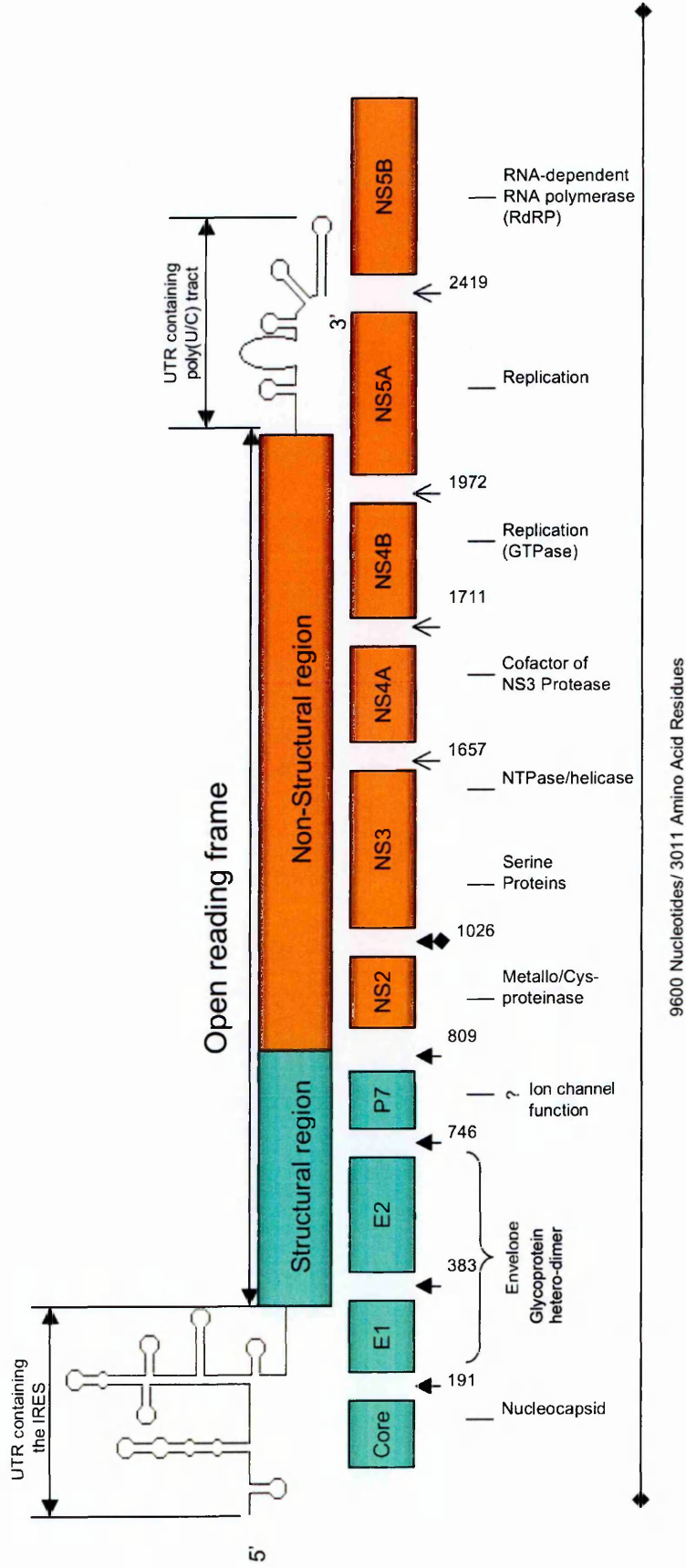
#### **1.4. Viral replication**

Current knowledge about HCV replication strategy is derived mainly from studies using *in vitro* recombinant systems involving the expression of HCV proteins as well as the recently developed cell-based system for subgenomic HCV RNA (replicon) replication. In general, it is thought that HCV follows a replication strategy similar to that of other positive-stranded RNA viruses (Pogue et al., 1994). HCV enters the target cell probably via receptor-mediated endocytosis with the help of the envelope glycoproteins E1 and E2 and HCV receptors on the cell surface. Accumulating evidence points to a role for CD81 in HCV entry. One study found that CD81 was required but not sufficient for cell entry of retroviruses pseudotyped with the HCV envelope glycoproteins E1 and E2, suggesting that CD81 is either a receptor or coreceptor for virus entry (Bartosch et al., 2003b; Zhang et al., 2004). A number of receptors have now been identified as being involved in viral attachment, these include: the low density lipoprotein (LDL) receptor (Agnello et al., 1999; Andre et al., 2002), Scavenger-R (Scarselli et al., 2002), DC-Sign and L-Sign (Cormier et al., 2004a; Lozach et al., 2004; Lozach et al., 2003). However, none of these receptors are unique to hepatocytes, and therefore can not explain the hepatotropism.

Upon cell entry, HCV undergoes uncoating, a process that is likely mediated by the viral envelope glycoproteins E1/E2 and results in fusion between viral and cellular membranes entry (Bartosch et al., 2003a; Bartosch et al., 2003b; Zhang et al., 2004). Following virus entry and uncoating, HCV RNA is released and initially serves as a mRNA for translation of the viral polyprotein. Subsequently, the HCV polyprotein is proteolytically processed into individual mature viral proteins. Co- and post-translational cleavage releases the non-structural (NS) proteins that are thought to form a replication complex, whereby NS5B is the central component of the HCV replication complex responsible for RNA synthesis during RNA replication (Tu et al., 1999).

Within this replication complex, HCV RNA replication is then initiated by synthesis of complementary negative-strand RNA, which in turn acts as a template for replication of more positive-strand RNA genome. Finally, nascent HCV RNA genome and viral structural proteins are assembled to form virions that egress from the infected cell.

However, replication is also dependent on both the 5' and 3' UTR's. The 3' UTR is composed of a variable region (VR), a poly U/C tract and a highly conserved 98-nucleotide sequence at the 3' end (Kolykhalov et al., 1996; Tanaka et al., 1996). The latter two regions have been shown to be essential for HCV RNA replication (Kolykhalov et al., 2000; Yanagi et al., 1999). The variable region, consisting of 2 stem loops, has been shown not to be essential for replication, but its absence reduces the replication efficiency (Friebe and Bartenschlager, 2002). The 5' UTR consists of 4 stem loops, of which 1a and 1b have been shown to be important in the generation of the negative RNA strand (Friebe et al., 2001).



**Figure 1.1. HCV genome organisation and encoded proteins.** The ORF is translated to produce one polypeptide that is cleaved by host proteases (thin arrows) and by viral proteases (thick arrows). NS2 and NS3 are responsible for auto-cleavage (arrow+rectangle).



### **1.5. Pathogenesis and disease outcome**

Infection with HCV results in the establishment of chronic viraemia in the majority of infected persons. Acute HCV infection is often asymptomatic and thus rarely diagnosed; however symptoms are documented in some individuals with acute HCV infection, and include jaundice, malaise, and nausea (Seeff, 2002; Seeff and Hoofnagle, 2002). HCV infects hepatocytes, as well as potentially B lymphocytes and other cells such as DCs (Okuda et al., 1999; Zignego et al., 1995), with high rates of viral replication estimated at more than 10 trillion virions produced per day during chronic infection (Neumann et al., 1998). Current estimates indicate that 50% or more of hepatocytes are infected with HCV (Agnello et al., 1998). Although a number of host factors play a role in determining disease outcome, most chronic infections will lead to hepatitis and some degree of fibrosis. Cirrhosis is estimated to occur in 15-20% of infected individuals, and approximately 1-4% of these may develop hepatocellular carcinoma after many years or decades of chronic infection (Alter and Seeff, 2000; Liang et al., 2000; Seeff, 2002; Seeff and Hoofnagle, 2002).

No vaccines for HCV currently exist. The current standard therapy for chronic HCV infection is pegylated interferon alpha with ribavirin (reviewed in reference Chander et al., 2002), and this leads to a sustained reduction in virus load in approximately 42% of genotype 1-infected individuals, and up to 80% in persons infected with genotype 2 or 3 (Chander et al., 2002; Feld and Hoofnagle, 2005; Manns et al., 2001).

Despite high rates of *in vivo* viral replication, efforts to grow HCV in culture have been largely unsuccessful (Bartenschlager and Lohmann, 2000; Chander et al., 2002; Grakoui et al., 2001), thus hindering the development of more effective anti-viral therapies. However, progress is currently being made in the study of HCV replication in cell culture with the development of selectable subgenomic HCV replicons (Bartenschlager and Lohmann, 2000; Blight et al., 2000; Lohmann et al., 2001; Lohmann et al., 1999); these include now systems which produce infectious progeny. Just recently, a full-length HCV genome able to replicate and produce virus particles that are infectious in cell culture has been described (Lindenbach et al., 2005).

## **2. General Immunology: Cell types needed for protective immune responses**

### **2.1. Dendritic cells and the priming of a naïve T-cell response**

Dendritic cells (DCs) function as “professional” antigen-presenting cells. They coordinate and regulate T-cell responses and are the only cells known to efficiently prime naïve T-cells. DCs exist in low numbers in peripheral tissues where they constantly sample their environment by phagocytosis, micropinocytosis and receptor-mediated endocytosis. The most abundant receptors for this are C-type lectins which recognise glycosylated antigens (Hart, 1997). Immature DCs constitutively migrate to draining lymph nodes where they do not stimulate T-cell responses. However, DCs may be activated in the peripheral compartments and stimulated to undergo maturation.

Multiple stimuli that signal infection or inflammation can stimulate DC maturation.

DC maturation entails the up-regulation of MHC and co-stimulatory molecules on the cell surface (a process that will enhance the DC/T-cell interaction), a decrease in antigen internalisation (which limits antigen sampling to DC's in the periphery), and profound changes in DC morphology and chemokine receptor expression, which prompts their migration to secondary lymphoid organs. Here mature DCs encountering naïve T-cells will orchestrate an appropriate T-cell response.

DCs have some specific features that make them particularly effective in priming a naïve T-cell response. Exogenous antigens associate with MHC-class II peptides in class II vesicles formed by the fusion of endosomes and exocytic vesicles. In DCs, unlike other APCs, formation and transport to the cell surface of MHC-class II-peptide complexes is rapidly induced by maturation (Cella et al., 1997). Like other cells, DCs can also present endogenous antigens to CD8<sup>+</sup> T-cells. However this process is again facilitated by DC maturation which up-regulates HLA class I expression. A number of studies have suggested that DC maturation may be subverted in HCV infection.

In many situations however, effective CD8<sup>+</sup> T cell responses need to be generated against endogenous antigens that are produced in cells other than DCs. This is likely to be the case for HCV infection where viral replication takes place predominantly in hepatocytes (Fig. 1.2). There is some evidence that in fact hepatocytes can prime a T-cell response, but that these T-cells rapidly undergo apoptosis (Bertolino et al., 1998). The transfer of these endogenous antigens to APCs via an exogenous route, and the presentation of these antigens to CD8<sup>+</sup> T-cells, is a process referred to as cross-presentation.

The intracellular pathway for loading of peptides during cross-presentation is currently not clear (Yewdell et al., 1999). Interestingly it has recently been shown that the cross-priming of CD8<sup>+</sup> T-cells by DCs is stimulated by IFN- $\alpha$  in a murine model (Le Bon et al., 2003).

However, mature DCs do not simply stimulate T-cells in a uniform fashion. Importantly, there is increasing evidence that the nature of the DC maturation factor (Reis e Sousa et al., 1999), the cytokine milieu in which this takes place (De Smedt et al., 1997; Hochrein et al., 2000) and the duration of the maturation stimulus (Langenkamp et al., 2000) influence the nature and the polarisation of the primed T-cell response.

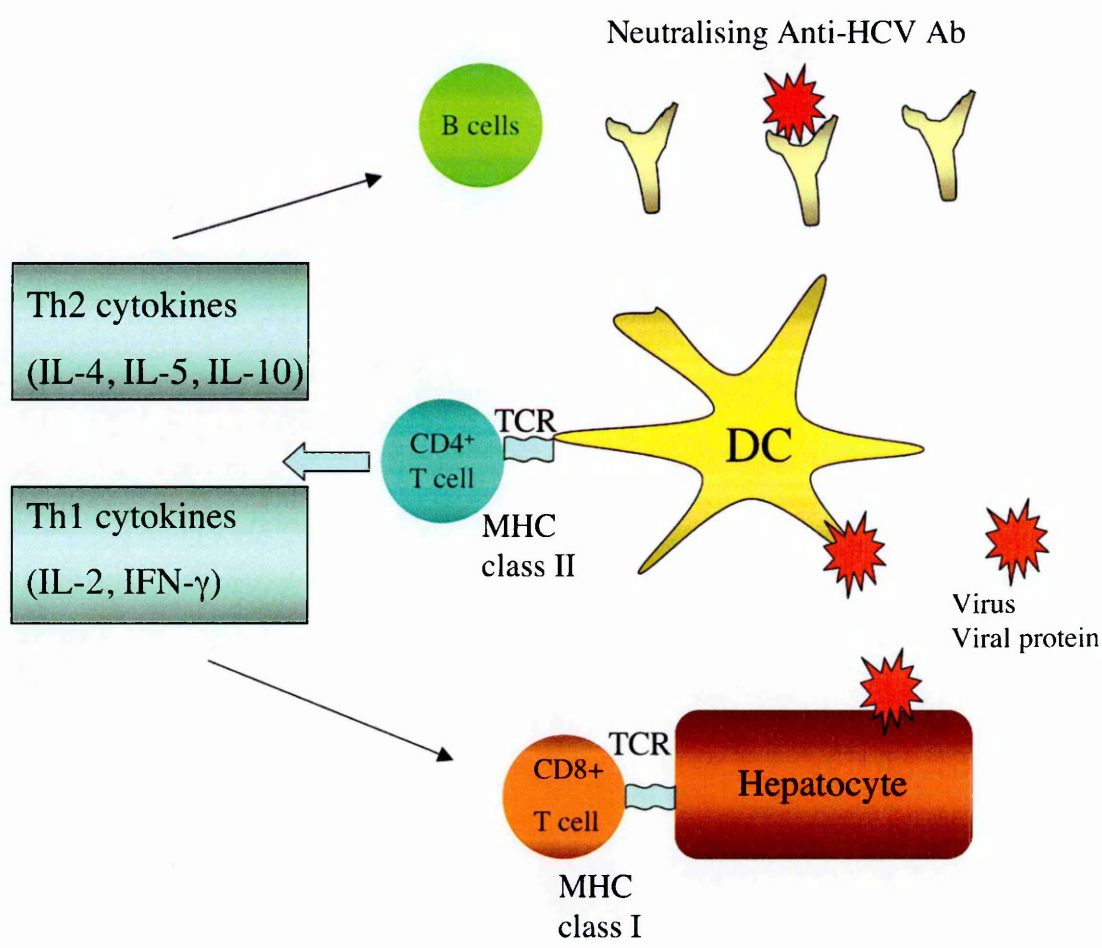


Fig. 1.2. Immune responses to HCV

## 2.2. Antigen recognition by CD4<sup>+</sup> T cells

CD4<sup>+</sup> and CD8<sup>+</sup> T-cells recognise antigens presented in association with MHC gene products. Generally speaking, exogenous proteins are degraded in endosomes and presented in conjunction with MHC class II to the  $\alpha\beta$ TCR on CD4<sup>+</sup> T cells. MHC Class II expression and therefore antigen presentation to CD4<sup>+</sup> T cells is restricted to certain cell types: DCs, monocytes and B cells. Other cell types, including it is thought hepatocytes, can express MHC class II in response to inflammatory stimuli (Cruickshank et al., 1998). This contrasts with endogenous proteins and peptides, found in the cytosol, and which are presented in conjunction with MHC class I (which is expressed on all cell types) to the  $\alpha\beta$ TCR on CD8<sup>+</sup> T cells.

Antigen recognition by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells results in cytokine production which further promotes clonal expansion and which can induce a wide range of effector mechanisms such as NK activation and the promotion of CD8<sup>+</sup> T cell maturation into cytotoxic T cells (CTL). CTL-mediated killing involves two complementary mechanisms: the perforin and the granzyme B pathways. Exogenous antigens are endocytosed by APC where they are processed by lysosomal proteases to generate peptides that can be presented by MHC class II molecules (Hunt et al., 1992; Morrison et al., 1986). Newly synthesised MHC class II  $\alpha\beta$  dimers associate with chaperone Invariant chain (Ii) in the endoplasmic reticulum, where the class II-associated invariant chain peptide (CLIP) is inserted into the peptide-binding groove of the  $\alpha\beta$  dimers (Cresswell, 1994).

Upon exiting the endoplasmic reticulum,  $\alpha\beta$ li complexes transverse the Golgi and can directly enter the endocytic compartments whereby li is proteolytically cleaved (Chapman, 1998). In humans, HLA-DM/HLA-DO (H-2DM/H-2DO in mice) then catalyzes the substitution of antigenic polypeptides for CLIP (Liljedahl et al., 1998) to generate MHC class II/peptide complexes and transported to the cell surface. Unlike MHC class I molecules that have a restricted binding site that accommodates peptides of 8-10 residues (Bjorkman et al., 1987; Jardetzky et al., 1991; Madden et al., 1991), (Bjorkman et al., 1987; Jardetzky et al., 1991; Madden et al., 1991), the binding site of MHC class II molecules can accommodate peptides of various sequences and lengths, thereby allowing presentation of a large spectrum of antigenic peptides using only a limited number of MHC class II molecules (Chicz et al., 1993; Rudensky et al., 1991).

CD4<sup>+</sup> T cells arise from thymic lymphoid progenitor cells that differentiate in the thymus and ultimately express a specific heterodimeric T cell antigen receptor (TCR) composed of an  $\alpha$  and  $\beta$  chain (Raulet et al., 1985; Samelson et al., 1985; Snodgrass et al., 1985a; Snodgrass et al., 1985b). The diverse repertoire of TCRs capable of recognising an enormous array of antigens arises from somatic rearrangement of multiple gene segments, including several variable (V) segments, diversity gene segments (D) and joining segments (J) in the case of  $\beta$  chains, which form the variable region of the TCR (Akira et al., 1987; Yancopoulos et al., 1986). Based on the number of V, D, and J segments, estimates are that  $10^{15}$  different amino acid sequences of TCRs can be potentially generated by V(D)J recombination (Davis and Bjorkman, 1988), thus allowing for recognition of any antigen encountered.

Activation and subsequent proliferation of CD4<sup>+</sup> T cells requires binding of the TCR/CD3 complex to the peptide/MHC class II complex (Littman, 1987), which is further stabilised by binding of CD4 to the MHC class II molecule as well as engagement of costimulatory molecules such as CD28 and B7. Naïve CD4<sup>+</sup> T cells encounter antigen bound to MHC class II molecules on dendritic cells in the T cell areas of lymphoid organs (Banchereau and Steinman, 1998). Upon antigen recognition by CD4<sup>+</sup> T cells, a cascade of intracellular signalling pathways is triggered which ultimately leads to the activation of transcription factors, including NFκB, NFAT, and AP-1, that initiate new gene transcription resulting in the differentiation, proliferation, and effector action of CD4<sup>+</sup> T cells (Acuto and Cantrell, 2000; Alberola-Ila et al., 1997; Crabtree, 1989; Murphy and Reiner, 2002). Following clonal expansion and acquisition of effector functions of antigen-specific CD4<sup>+</sup> T cell populations, most of the activated T cells die by apoptosis in a contraction phase (Abbas, 1996; Dutton et al., 1998; Lenardo et al., 1999; Russell, 1995), and a subset of T cells activated during the primary response persists as a long-lived population of memory CD4<sup>+</sup> T cells.

A major consequence of CD4<sup>+</sup> T cell activation is the production of interleukin (IL)-2 and synthesis of the α chain of the IL-2 receptor, which associates with the β and γ chains expressed on resting T cells to form a heterotrimeric receptor with a high affinity for IL-2. Binding of IL-2 to its high affinity receptor drives further proliferation and differentiation of CD4<sup>+</sup> T cells into effector cells. Furthermore, activation of CD4<sup>+</sup> T cells results in the expression of CD40 ligand (CD40L), which binds to CD40 that is expressed on B cells, dendritic cells, and macrophages (Caux et al., 1994; Xu et al., 1994).



Ligation of CD40 by CD40L activates B cells to produce antibodies (Kawabe et al., 1994; Kennedy et al., 1994), and stimulates macrophages to produce TNF- $\alpha$ , nitric oxide, and IL-12 and to express co-stimulatory receptors (Alderson et al., 1993; Shu et al., 1995; Stout et al., 1996; Tian et al., 1995). Finally, ligation of CD40 on dendritic cells results in upregulation of expression of MHC molecules and co-stimulatory molecules, as well as increased production of cytokines, especially IL-12 (Caux et al., 1994; Cella et al., 1996). Once activated, these APCs can function to prime antigen-specific CTL responses.

### **2.3. The generation of effector and memory T cell subsets**

Once a naïve CD4<sup>+</sup> or CD8<sup>+</sup> T-cell has been primed, activation through the T-cell receptor (TCR) and co-receptors results in proliferation and the acquisition of effector functions that ultimately produce an array of effector and memory T-cell subsets. The pathways that generate these cell types are controversial (Baron et al., 2003; Geginat et al., 2001; Wherry et al., 2003b), but it is clear that these cells differ in their capacity to proliferate in response to antigen, the cytokines that they produce and their cytotoxic functions. The most studied T-cell subsets so generated are the CD4<sup>+</sup> T helper 1 (which produce predominantly IFN- $\gamma$ ) and T helper 2 subsets, which produce predominantly IL-4 and IL-5. However, the Th1/Th2 paradigm is almost certainly over-simplistic (Woodland and Dutton, 2003), but followed in the discussion here:

During their initial encounter with antigen, CD4<sup>+</sup> T cells differentiate into either type 1 (Th1) or type 2 (Th2) cells, which differ in the cytokines produced upon stimulation, and thus differ in their function.

Cytokines themselves are the most potent inducers of differentiation of naïve CD4<sup>+</sup> T cells into either Th1 or Th2 cells. The principle Th1-inducing cytokine is IL-12, produced by activated macrophages and dendritic cells (Szabo et al., 1995), while IL-4 drives the differentiation of Th2 cells (Seder and Paul, 1994). IL-12 and IL-4 activate distinct signal transducer and activator of transcription (STAT) proteins (STAT4 and STAT6 respectively) that induce differentiation of these cells to Th1 and Th2 populations (Abbas et al., 1996; Murphy and Reiner, 2002). Furthermore, the two subsets of T-helper cells can regulate each other, for instance IL-10 produced by Th2 cells can inhibit the development of Th1 cells (Fiorentino et al., 1989), while IFN- $\gamma$  produced by Th1 cells can prevent the activation of Th2 cells (Chung, 2001; Fitch et al., 1993). Th1 cells produce predominantly IFN- $\gamma$ , TNF- $\alpha$ , and IL-2, whereas Th2 cells produce cytokines such as IL-4, IL-5, and IL-13 (Abbas et al., 1996; Mosmann et al., 1986; Murphy et al., 2000). Th1 and Th2 subsets were however originally defined in mice (Mosmann et al., 1986), and with the advent of more sensitive assays for cytokine production, it has become clear that memory T cells in many immune responses, especially in humans, display complex patterns of cytokine production that are not easily classified as Th1- or Th2-type; T-helper cells that can produce both Th1 and Th2 cytokines have been designated Th0 (reviewed in reference (Abbas et al., 1996).

IFN- $\gamma$ , the principal effector cytokine produced by Th1 cells, activates macrophages and increases expression of MHC class I and class II molecules (Dalton et al., 1993), and can activate B cells to produce strongly opsonising antibodies belonging to certain immunoglobulin G (IgG) subclasses (IgG1 and IgG3 in humans) (Finkelman et al., 1988; Snapper and Paul, 1987; Stevens et al., 1988).

IFN- $\gamma$  and IL-2 production also promote the differentiation of CD8<sup>+</sup> T cells into active cytotoxic cells. Furthermore, IFN- $\gamma$  can also have direct anti-viral effects on cells by inducing cellular proteins such as double-stranded RNA activated protein kinase (PKR), 2'-5'-oligoadenylate synthetase, dsRNA-specific adenosine deaminase (dsRAD), and MxA that can decrease viral transcription and/or replication (Boehm et al., 1997; Haller et al., 1998; Horisberger et al., 1983; Mestan et al., 1986; Rebouillat and Hovanessian, 1999).

IL-4 and IL-5 are the key cytokines produced by Th2 cells. IL-4 stimulates B cells to produce IgE, thereby initiating IgE-dependent, mast cell-mediated reactions (Galli, 1993; Mosmann and Coffman, 1989). Th2 cytokines also provide help to B cells to produce high levels of IgM and non-complement-fixing IgG isotypes, including IgG1 in mice and IgG4 in humans (Coffman et al., 1993; Del Prete et al., 1991). Additionally, IL-5 can increase the production of eosinophils, thus promoting allergic responses effective in eliminating parasitic infections (reviewed in reference (Mahanty and Nutman, 1993). Several Th2 cytokines have anti-inflammatory properties (Abbas et al., 1996), suggesting that Th2 cells may function as regulators to limit the pathologic consequences of Th1-mediated protective immunity.

### **3. Phenotypic analysis of functional subsets of CD4<sup>+</sup> T cells in viral infections**

A hallmark of the immune response is the establishment of immunological memory, allowing lifelong immunity against reinfection. Subpopulations of memory T cells that are distinguishable by surface phenotypic markers have been known to exist for some time (Doherty et al., 1996), although more recently subpopulations of memory T cells, designated 'effector' memory and 'central' memory, have been proposed based on the differential expression of CCR7 (Lanzavecchia and Sallusto, 2005; Sallusto et al., 2004; Sallusto et al., 1999), a chemokine receptor that, when engaged to its ligand SLC expressed on endothelial cells (Gunn et al., 1998), promotes adhesion and transmigration of T cells into lymph nodes (Butcher and Picker, 1996; Campbell et al., 1998). Central and effector memory T cells were defined as two functionally distinct subsets for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in humans. Central memory CD4<sup>+</sup> T cells were defined as CCR7<sup>+</sup>/CD45RA<sup>-</sup>/CD62L<sup>hi</sup>, whereas effector memory CD4<sup>+</sup> T cells were defined as CCR7<sup>-</sup>/CD45RA<sup>-</sup>/CD62L<sup>lo</sup> (Lanzavecchia and Sallusto, 2005; Sallusto et al., 2004; Sallusto et al., 1999). The reciprocal expression of CD45RA and CD45RO isoforms has been used to identify naïve and memory T cells respectively (Michie et al., 1992), while CD62L (L-selectin) is another receptor expressed on T cells that is essential for lymphocyte migration to lymph nodes (Butcher and Picker, 1996).

In the initial report identifying two subsets of central and effector memory cells in humans, different effector functions as measured by cytokine production following polyclonal stimulation were reported for these subsets, with central memory T cells producing predominantly IL-2, and effector memory cells producing high amounts of IFN- $\gamma$ , IL-4, and IL-5 (Lanzavecchia and Sallusto, 2005; Sallusto et al., 2004; Sallusto et al., 1999). However, in a recent study of acute LCMV infection in mice, LCMV-specific effector memory and central memory CD8<sup>+</sup> T cells isolated on the basis of CD62L expression were reported to have similar capacities to produce IFN- $\gamma$  and TNF- $\alpha$ , although only central memory cells were able to produce IL-2 (Wherry et al., 2003a), consistent with previous findings in human systems (Sallusto et al., 1999). Furthermore, adoptive transfer of populations of LCMV-specific central memory or effector memory CD8<sup>+</sup> T cells in mice demonstrated that, in the absence of antigen, effector memory cells convert to central memory cells, and upon reencounter with antigen, central memory cells revert back to effector memory (Wherry et al., 2003a). These data from a murine acute viral infection model suggest central memory and effector memory T cells are not necessarily distinct subsets, but rather part of a continuum in a linear differentiation pathway (Wherry et al., 2003a). Together these data suggest that central memory cells represent an antigen-primed population that travels to secondary lymphoid structures and is capable of expansion and activation upon secondary encounter with antigen, whereas effector memory cells can enter peripheral tissues to contain invasive pathogens.

The host species and nature of infection (chronic persistent viral infection or acute infection that is resolved) are likely to influence the resulting populations of antigen-specific effector and central memory T cells generated, and it remains to be determined whether these subsets represent distinct lineages in humans, or are part of a linear pathway of differentiation that can convert from one subtype to the other depending on the presence of antigen as demonstrated in murine models (Wherry et al., 2003a).

Since the initial description of different subsets of memory T cells in bulk CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations (Sallusto et al., 1999), numerous studies have applied these phenotypic characteristics to the study of virus-specific T cells in human viral infections, most notably virus-specific CD8<sup>+</sup> T cell responses as the use of MHC class I tetramers greatly facilitates the identification of specific populations of T cells for phenotypic analysis (Appay et al., 2002; Champagne et al., 2001; Ellefsen et al., 2002; Urbani et al., 2002). These studies have suggested that a defect in the maturation of virus-specific CTL responses based on CD45RA and CCR7 expression may exist in individuals infected with persistent uncontrolled viruses such as HIV (Champagne et al., 2001). However, further studies of the phenotype of CD8<sup>+</sup> T cells specific for HIV, EBV, CMV, and HCV in acute and chronic stages of infection suggest that the phenotype of virus-specific CD8<sup>+</sup> T cells may differ according to epitope specificity as well as disease state, thus making it more difficult to ascribe specific functions to subsets of T cells with different phenotypes (Appay et al., 2002).

Similar studies of the phenotype and function of virus-specific CD4<sup>+</sup> T cells in humans have been more difficult to perform due to the lack of sensitive and specific MHC class II tetramers available for their *ex vivo* analysis.

However, a limited number of studies have combined intracellular staining for IFN- $\gamma$  production with surface staining for CCR7, CD45RO, and CD62L expression to determine the phenotype of virus-specific CD4<sup>+</sup> T cells in HIV and CMV infections (Harari et al., 2002; Sester et al., 2002). CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells from HIV-positive subjects stimulated with recombinant p55 Gag protein were predominantly CCR7<sup>-</sup> (Harari et al., 2002); likewise CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> cells from CMV-positive subjects stimulated with CMV lysate antigens were CD45RO<sup>+</sup>/CD62L<sup>-</sup>/CCR7<sup>-</sup> (Sester et al., 2002). Taken together, these results indicate these HIV- and CMV-specific CD4<sup>+</sup> T cells belong to a subset of mature effector cells. However, future studies are warranted to further characterise the relationship between phenotypic markers and effector functions of virus-specific CD4<sup>+</sup> T cells that may provide insight into their role in the outcome of human viral infections.

#### **4. Methods for detection and quantification of antigen-specific CD4<sup>+</sup> T cells**

Our understanding of antigen-specific CD4<sup>+</sup> T cells has been largely dependent on the methods available for their detection and quantification in the host.

The conventional assay is the lymphocyte proliferation assay (LPA), in which peripheral blood mononuclear cells (PBMC) are cultured in the presence of recombinant antigens. After 6 days, lymphocyte proliferation is measured by pulsing with radiolabelled nucleotide (<sup>3</sup>H-thymidine) (Corradin et al., 1977).

Results are expressed as either the difference in counts per minute (CPM) in the presence and absence of antigen ( $\Delta$ CPM), or a ratio of the CPM in the presence of antigen divided by the CPM in the presence of control antigen (termed stimulation index, or SI). LPAs thus provide important insight into which proteins are immunogenic in a viral infection, although disadvantages are that LPAs do not provide a direct measurement of the frequency of virus-specific CD4<sup>+</sup> T cells *ex vivo*, nor do they address the breadth of specific viral epitopes targetted within each protein by the virus-specific CD4<sup>+</sup> T cell response.

An alternative to measuring proliferation by incorporation of radiolabeled thymidine is the use of fluorescent dyes such as 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), which spontaneously and irreversibly couples to both intracellular and cell surface proteins. Upon cell division, CFSE labeling is equally distributed to the progeny cells, thus the fluorescence intensity is reduced by half with each cell division and can be readily followed by flow cytometry; CFSE can successfully measure approximately 7 to 10 cell divisions (Lyons, 1999; Lyons and Parish, 1994). An advantage of CFSE labelling versus pulsing with <sup>3</sup>H-thymidine is that CFSE can be used in conjunction with other fluorescent antibodies for analysis by flow cytometry, thus allowing for assessment of proliferation of certain subsets of T cells by co-staining with tetramers or cytokines such as IFN- $\gamma$ . Examples of the use of CFSE in assessing proliferation of virus-specific T cells in humans include a study by Migueles *et al* analyzing proliferation of virus-specific CD8<sup>+</sup> T cells in HIV-infected individuals by CFSE-labeling PBMC in conjunction with HIV and cytomegalovirus (CMV) MHC class I tetramers (Migueles *et al.*, 2002).



In terms of virus-specific CD4<sup>+</sup> T cell responses, CFSE labelling of p24-stimulated PBMC from subjects with HIV infection in conjunction with staining for IFN- $\gamma$  production has been described as a means for determining correlations between cellular proliferation and effector cytokine production by virus-specific CD4<sup>+</sup> T cells (McNeil et al., 2001; Palmer et al., 2002).

A more recently developed method for the measurement of virus-specific CD4<sup>+</sup> T cells is the single cell enzyme-linked immuno spot (ELISpot) assay. In this case, the magnitude and frequency of CD4<sup>+</sup> T cells can be measured by stimulation in an overnight assay with specific viral peptides and subsequent production of cytokines, most commonly IFN- $\gamma$ , that are detected by enzyme-linked anti-cytokine antibodies and visualised by the formation of blue spots in wells of a 96-well nitrocellulose plate (Czerkinsky et al., 1988). ELISpot assays for detection of CD4<sup>+</sup> T cells have been used in multiple systems for both murine and human viral infections (Christensen and Doherty, 1999; Leen, 2001; Steele et al., 2002; Topham and Doherty, 1998; van Bleek et al., 2003; Whitmire et al., 1998). However, CTL epitopes could be contained within the peptides tested; therefore care should be taken to deplete CD8<sup>+</sup> T cells before putting PBMC into ELISpot assays to confirm that those positive responses seen are in fact due to CD4<sup>+</sup> T cells.

The development of flow cytometric-based assays has greatly advanced the ability to visualise and quantify antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

Intracellular cytokine staining (ICS) has been developed for murine viral infections, including LCMV (Kamperschroer and Quinn, 1999; Varga and Welsh, 1998; Whitmire et al., 1998), as well as human viral infections such as CMV and HIV (Kern et al., 2002; Maecker et al., 2001; Pitcher et al., 1999; Rentenaar et al., 2000; Waldrop et al., 1997). Like ELISpot assays, in that cytokine production is measured, standard ICS protocols depend on stimulation of PBMC with viral peptides or recombinant proteins for a shorter period of time (usually 6 hours), and require the addition of a protein secretion inhibitor such as brefeldin A to allow for the intracellular accumulation of cytokines. Advantages of ICS include the ability to stain for cytokine production in conjunction with antibodies to multiple different surface markers, including activation markers and regulatory markers that may provide further insight into the functions of these cells. Frequencies of antigen-specific CD4<sup>+</sup> T cells of less than 0.05% of total CD4<sup>+</sup> T cells may however preclude direct detection of low-frequency virus-specific CD4<sup>+</sup> T cells without further *in vitro* expansion.

Perhaps the greatest advance in the study of virus-specific T cells has come from the development of tetrameric MHC/peptide complexes, which were initially developed using MHC class I alleles and class I restricted peptides (Altman et al., 1996; Ogg, 2000). Previous methods as described above for the measurement of antigen-specific lymphocytes had relied on a functional activity, such as proliferation or cytokine production, as a measurement of virus-specific T lymphocyte activity. Class I tetramers allow for the direct detection of virus-specific CD8<sup>+</sup> T lymphocytes and can thus identify populations of cells that are lacking in effector function (Zajac et al., 1998).

The use of MHC class I tetramers suggested that such dysfunctional populations of virus-specific CD8<sup>+</sup> T cells do exist in chronic human viral infections such as HCV and HIV (Champagne et al., 2001; Gruener et al., 2001; Kantzanou et al., 2003; Klenerman et al., 2002a). Class II tetramers are less well described, and the majority of published reports have been restricted to the analysis of CD4<sup>+</sup> T cell lines or clones (Crawford et al., 1998; Kwok et al., 2000; Meyer et al., 2000; Novak et al., 1999a). Technological advances in the sensitivity and specificity of class II tetramers are rapidly being made, and these tetramers will likely prove useful tools for the analysis of virus-specific CD4<sup>+</sup> T cells.

## **5. CD4<sup>+</sup> T cell immune responses in HCV infection**

Although different subsets of cells in the immune system are involved in HCV infection, I will be primarily focusing on the CD4<sup>+</sup> T cell response, as this has been the main field of study during my PhD.

Cellular immune responses, involving both CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) and CD4<sup>+</sup> T-helper cells, play an essential role in the control of HCV infection. Whereas CTLs are thought to be the main effector cells that eliminate HCV-infected cells, HCV-specific CD4<sup>+</sup> T cells play a critical role in providing help by cytokine production and activation of antigen-presenting cells that is required for maintenance of effective virus-specific memory CTL responses. Antibody-mediated depletion of CD4<sup>+</sup> T cells before reinfection of two immune chimpanzees resulted in persistent, low-level viraemia despite functional intra-hepatic memory CD8<sup>+</sup> T cell responses (Grakoui et al., 2003).

Incomplete control of HCV replication by memory CD8<sup>+</sup> T cells in the absence of adequate CD4<sup>+</sup> T cell help was associated with emergence of viral escape mutations in class I MHC-restricted epitopes and failure to resolve HCV infection, showing the importance of requirement for CD4<sup>+</sup> T cell help to control HCV re-infection (Grakoui et al., 2003). In addition, cytokine production by CD4<sup>+</sup> T cells stimulates B cell activation and subsequent production of antibodies. Current data on the function and role of virus-specific CD4<sup>+</sup> T-helper cells in acute and chronic HCV infection will be reviewed here.

### **5.1. CD4<sup>+</sup> T cell responses in acute HCV infection**

CD4<sup>+</sup> T cell responses in the liver of acute HCV infection in humans have not been characterised to date. In chimpanzees, liver CD4<sup>+</sup> T cells have been expanded with anti-CD3 and IL-2 and then tested for proliferation in response to HCV proteins (Thimme et al., 2002). In those who failed to control virus, no CD4<sup>+</sup> T cell responses were identified whereas in those chimps who transiently or permanently controlled the virus strong proliferative CD4<sup>+</sup> T cell responses were detectable. However, CD4<sup>+</sup> T cell responses are not absent in all acute infections that persist. Acute resolving HCV infection was associated with a sustained response by HCV-specific CD4<sup>+</sup> T cells in one representative study (Gerlach et al., 1999). Here, patients who failed to clear the virus were divided into 2 groups: group 1 was unable to mount an HCV-specific CD4<sup>+</sup> T cell response and developed chronic HCV. In group 2 HCV RNA was cleared initially and was associated with strong HCV-specific CD4<sup>+</sup> T cell responses. However, these responses diminished just before a rebound of viraemia that resulted in chronic infection.

Therefore, a vigorous anti-viral CD4<sup>+</sup> T cell response (as measured by proliferation assays) in the early and late phase of acute HCV seems necessary to achieve long-term viral control (CD4<sup>+</sup>/Th1 response).

Another group has also shown (Missale et al., 1996) that the vigour of the T cell response during the early stages of infection may be a critical determinant of disease resolution and control of infection.

That persistent infection can develop despite the presence of acute-phase HCV-specific CD4<sup>+</sup> T cell responses has been shown in a study of healthcare workers exposed to needle-stick injuries (Thimme et al., 2001): although 2 individuals had strong HCV-specific CD4<sup>+</sup> T cell proliferative responses in the acute phase with significant decreases in HCV RNA initially, they subsequently became chronically infected.

Evolution of the infection to chronicity can be explained by the finding that HCV-specific CD4<sup>+</sup> T cells may survive initially despite failing to proliferate or produce IFN- $\gamma$ , suggesting anergy or exhaustion, and diminish eventually as infection persists (Ulsenheimer et al., 2003).

Overall, these studies suggest that a range of HCV-specific CD4<sup>+</sup> T cell responses in the acute HCV phase can exist in blood and liver despite the fact that individuals eventually become chronically infected, but these responses are not sustained. Why and how HCV manages to inactivate CD4<sup>+</sup> T cells remains an important question in understanding its persistence.

### ***5.1.1. MHC class II restricted epitopes***

Permanent resolution of infection is related to the breadth (number of CD4 epitopes) and magnitude of HCV-specific CD4<sup>+</sup> T cell responses. NS3 protein has been shown to be one dominant target of CD4<sup>+</sup> T cell responses in humans clearing HCV infection (Diepolder et al., 1995b). Some epitopes in NS3 have been identified in individuals and chimps with resolved infection (Day et al., 2002; Diepolder, 1997; Lamonaca et al., 1999; Shoukry et al., 2004), and numerous studies indicate that CD4<sup>+</sup> T cells targeting most HCV proteins are the norm in self-limited infections (Day et al., 2002; Gerlach et al., 1999; Lechner et al., 2000; Rosen et al., 2002; Takaki et al., 2000; Thimme et al., 2002; Thimme et al., 2001).

The close relationship between a polyclonal, multispecific proliferative CD4<sup>+</sup> T cell response that needed to be sustained and is directed mainly against the NS proteins of virus and viral clearance in acute HCV has been also shown by other groups (Diepolder et al., 1995a; Missale et al., 1996).

The number of CD4<sup>+</sup> T cell epitopes recognised during acute HCV infection has been estimated by characterising memory CD4<sup>+</sup> T cell populations in blood after permanent resolution of the virus: one study showed at least 4, and up to 14 epitopes from the core, NS3, NS4 and NS5 proteins were recognised by CD4<sup>+</sup> T cells in patients several months or even years after loss of HCV RNA (Day et al., 2002). A recent study identified 13 CD4<sup>+</sup> T cell epitopes within the NS3-NS4 region that were recognised by  $\geq 30\%$  of patients with acute or resolved HCV (Gerlach et al., 2005).

Of these, eight peptides were also recognised recurrently from different donors by specific CD4<sup>+</sup> T cell clones in independent cloning procedures.

Multispecific CD4<sup>+</sup> T cell responses were also detectable in blood of individuals during acute HCV infection acquired by needlestick injury (Thimme et al., 2001) or iv drug use (Lechner et al., 2000).

Importantly, in some patients whose infection became chronic after the acute phase infection, responses were similar to those who spontaneously cleared the virus after acute infection (Thimme et al., 2001). The only difference was that these responses in individuals with chronic infection were not sustained.

Table 1.1 displays the most common described CD4<sup>+</sup> restricted epitopes.

Amino acid position	HCV Protein	AMINO ACID SEQUENCE	HLA-RESTRICTION	REFERENCE
aa 21- 40	Core	DVKFPGGGQIVGGVYLLPRR	DRB1*1101,DQB1*0301	Day, 2002
aa 31- 45	Core	VGGVYLLPRR GPRLG	DRB1*1101	Godkin, 2001
aa 141- 155	Core	GAPLGGAARA LAHGV	DRB1*1101	Godkin, 2001
aa 393- 410	E2	GFATQRLTSLFALGPSQK	DRB1*1101	Frasca, 1999
aa 1241-1260	NS3	PAAYAAQGYKVLVLNPSVAA	DRB1*15, DRB1*0301 +	Day, 2002
aa 1248-1261	NS3	GYKVLVLNPSVAAT	DR4, DRB1*1101	Wertheimer, 2003
aa 1248-1267	NS3	GYKVLVLNPSVAATLGFGAY	DQB1*0301	Lamonaca, 1999
aa 1251-1259	NS3	VLVLNPSVA	DRB1*1101,DRB1*1201, DRB1*0401, DRB1*1302	Day, 2002
aa 1384-1401	NS3	VIKGRHLIFCHSKKKCD	DRB1*15, DR15	Eckels. 1999
aa 1531-1550	NS3	TPAETTVRLRAYMNTPLPV	DRB1*0701	Day, 2002
aa 1581-1600	NS3	ENLPYLVA YQATVCARAQAP	DRB1*1001	Day, 2002
aa 1686-1705	NS4a	VVLSGKPAIIPDREVLYREF	DRB1*0301	Harcourt, 2003
aa 1746-1765	NS4b	IAPAVQTNWQKLETFWAKHM	DRB1*16 or DRB3*0202	Harcourt, 2003
aa 1767-1786	NS4b	NFISGIQYLAGLSTLPGNPA	DRB1*1104	Carlos, 2004
aa 1771-1790	NS4b	GIQYLAGLSTLPGNPAIASL	DRB1*0404	Day, 2002
aa 1907-1926	NS4b	GPGE GAVOWMNRLIAFASRG	DRB1*1104,DQB1*0501	Lamonaca, 1999
aa 2268-2282	NS5a	VSVPAEILRK SRRFA	DRB1*1101	Godkin, 2001
aa 2571-2590	NS5b	KGGRKPARLIVFPDLGVRVC	DRB1*0404,DRB1*0407	Day, 2002
aa 2841-2860	NS5b	ARMILMTHFFSVLIARDQLE	DRB1*1101	Day, 2002
aa 2941-2955	NS5b	CGKYLFNWAV RTKLK	DRB1*1101	Godkin, 2001
aa 2941-2960	NS5b	CGKYLFNWAVRTKLKLPIA	DRB1*1101	Day, 2002

**Table 1.1. HCV T helper epitopes** mapped within a region of 21 amino acids or less. The protein, the sequence and HLA restriction elements of the T helper epitopes are provided



### **5.1.2. Association between HLA class II alleles and infection outcome**

Some MHC class II alleles in humans have been associated with persistence or resolution of HCV infection: for example, HLA-DRB 1\*0701 has been shown to be associated with persistence in patients who were homogeneous in terms of gender, source of infection and ethnicity (Fanning et al., 2001).

In contrast, a number of other studies have shown a strong association between other HLA class II alleles and viral control. These alleles are HLA-DRB1\*0101, HLA-DRB1\*1101, and HLA-DQB1\*0301 (Alric et al., 1997; Minton et al., 1998; Thursz et al., 1999), HLA-DRB1\*1101 and HLA-DQB1\*0301 (these genes are in close linkage disequilibrium) have been associated with a sustained CD4<sup>+</sup> T cell response in acute HCV infection; these responses were stronger than in non-DQB1\*0301+ controls (Harcourt et al., 2001). However, our understanding of this association remains incomplete. Some of these HLA class II-restricted peptides have been identified through epitope prediction programs (Godkin et al., 2001), and the full repertoire of naturally presented peptides is not completely defined. A recent study showed that viral variation might play a role in determining the dominance of epitopes seen within a population. Here, an HLA DR11-restricted epitope (NS3 aa1248-1261) that is highly conserved within viral genotypes was found not to be the immunodominant response, despite being the most commonly recognised epitope for this HLA allele (Harcourt, 2004). Epitopes that can<sup>not</sup> tolerate amino acid substitutions may thus appear to be less dominant: their capacity for increased variability means that they may be recognised less frequently within individuals.

### **5.1.3. Th1/Th2 profile and association with outcome**

It has been shown in studies of acute disease that viral clearance is more likely to occur when PBMC of patients display a Th1 profile (IFN- $\gamma$  and IL-2). Those with Th2 profile (IL-4 and IL-10) were more likely to become chronic (Tsai et al., 1997)  $\times$  suggesting that this phenotype (Th1) generates more protective immune responses in HCV. Here, PBMC CD4<sup>+</sup> T-cell proliferation and cytokine secretion in response to a panel of recombinant HCV antigens were assayed in 17 patients with acute HCV. All six patients with self-limited disease had a significant CD4<sup>+</sup> T-cell proliferation to C22, E1, C100, C200, and NS5, running parallel with the antigen-stimulated secretion of IL-2 and IFN- $\gamma$ , but not with IL-4 and IL-10, indicating predominant Th1 responses.

Among the remaining 11 patients who developed chronicity, several cases showed specific CD4<sup>+</sup> T cell responses, but their stimulation index, antigen-stimulated IL-2 and IFN- $\gamma$  production were significantly lower than those of cases with recovery. Importantly, IL-4 and IL-10 (Th2 responses) were detectable in the group who developed chronicity. The data suggested that activation of Th2 responses in acute hepatitis C patients might play a role in the development of chronicity. Similar results have been shown in a group of Irish women who were infected with HCV genotype 1b after the administration of contaminated anti-D immunoglobulin in 1977/1978 (MacDonald et al., 2002).

To summarise: those individuals who fail to generate a detectable virus-specific T cell response in the blood, the liver or both are at highest risk of persistent HCV infection. Nevertheless, the generation of a cellular immune response in the acute phase of infection does not necessarily mean that the infection will be permanently controlled.

It is more important that the responses are sustained for weeks or months after control of virus replication to prevent rebound and establishment of persistent infection, and also that appropriate cytokine secretion is maintained.

## 5.2. CD4<sup>+</sup> T cell responses in chronic HCV infection

Once chronic HCV infection is established, cellular immune responses are rarely detectable using current technology - and when detectable appear to be directed against a few epitopes only, and lacking in markers of activation.

Analysis of HCV-specific CD4<sup>+</sup> T cell responses in chronic HCV infection using ELISpot or ICS showed responses at low frequency<sup>ing</sup> in blood and only targeted a limited number of epitopes (Day et al., 2002; Gerlach et al., 1999; Lechner et al., 2000; Rosen et al., 2002; Takaki et al., 2000; Ulsenheimer et al., 2003; Wertheimer et al., 2003). In an expanded analysis of responses to recombinant HCV proteins in persons with resolved infection, an average of 10 epitopes was targeted, whereas in persons with chronic viraemia never more than one epitope was targeted (Day et al., 2002).

The question, whether HCV-specific CD4<sup>+</sup> T cell responses persist in chronic infection or whether they lack function in terms of proliferation and cytokine production (IFN- $\gamma$ ), has been addressed by staining for expression of the IL-2 receptor  $\alpha$ -chain (CD25), which is an early marker of activation.

This study showed (Ulsenheimer et al., 2003) that a small proportion of CD4<sup>+</sup> T cells from the blood of chronically infected individuals did upregulate CD25 after stimulation with recombinant HCV proteins, but lacked the capability for proliferation and IFN- $\gamma$  production.

The best approach for detecting functionally impaired CD4<sup>+</sup> T cells in chronic HCV infection is the use of MHC class II tetramers. Using this technique, a correlation has been shown between the clinical outcome and the presence of circulating CD4<sup>+</sup> T cells directed against the virus (Day et al., 2003). Here, with the use of 3 HCV HLA class II tetramers, HCV-specific CD4<sup>+</sup> T cells could be detected in subjects who spontaneously resolved HCV viraemia, but not in those with chronic HCV infection, suggesting that HCV-specific CD4<sup>+</sup> T cell frequencies are very low in PBMC.

A further application of this technology for the analysis of intrahepatic CD4<sup>+</sup> T cells could shed more light on their differentiation state and functional impairment.

However, the expansion of HCV-specific CD4<sup>+</sup> T cell lines by repeated stimulation with recombinant antigens indicated that frequencies are not always low (Minutello et al., 1993; Penna et al., 2002; Schirren et al., 2000; Tsai et al., 1997; Ulmer et al., 1993). CD4<sup>+</sup> T cell lines were established from some persistently HCV-infected individuals after stimulation of lymphocytes from liver and blood. Even in chronic infection, responses were not necessarily absent from either tissues.

However, responses were higher in the liver when compared with the blood, suggesting that they may be sequestered at the site of viral replication and inflammation. Some of these CD4<sup>+</sup> cell line responses produced anti-inflammatory cytokines such as IL-4 and IL-10, which have been shown to promote viral persistence and slow progression of liver disease (Nelson et al., 2003).

The role of IL-10 as Th2 anti-inflammatory cytokine has been demonstrated *in vivo* in humans chronically infected with HCV (Nelson et al., 2003). Here, individuals with advanced fibrosis were treated three times a week with IL-10 for 12 months.

Administration of IL-10 resulted in a decreased number of IFN- $\gamma$ -secreting HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells. At the same time ALT levels as a marker of inflammation were reduced, indicating the role of IL-10 as anti-inflammatory cytokine. However, with the loss of specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells, HCV RNA levels were increased, suggesting that these same cells are responsible for viral control (Nelson et al., 2003).

CD4<sup>+</sup> Th2 cytokines induced in natural chronic HCV infection have also been described as “malfunctioning” in terms of being inappropriately secreted in the blood and therefore permitting continual re-infection of the liver.

A Th1 profile type has been described in a group of HCV-recovered individuals where HCV-specific CD4<sup>+</sup> T cells were maintained even after the elimination of the virus (Sugimoto et al., 2003). These cells were 10 times as frequent and targetted 4 times as many epitopes in resolved compared to chronically infected individuals.

One hypothesis why in chronic HCV the Th2 type is the predominant element is that dendritic cells from patients with chronic HCV infection have defective allostimulatory function, possibly due to inhibition of IL-12 (Bain et al., 2001; Fowler et al., 2003).

The latter cytokine is required for the induction of Th1 type cells. This dendritic cell dysfunction might result in biased T cell polarisation, which could favor, for example, a Th2-type response.

Overall, failure of CD4<sup>+</sup> T cells is a key factor in HCV persistence. To some extent this appears to be due to loss/deletion of antigen-specific cells. On the other hand there is some evidence that a change in function also occurs in persistent infection, although whether this is cause or effect requires further study.

### **5.3. HCV-specific CD4<sup>+</sup> responses and co-infection with HIV**

Persons with human immunodeficiency virus (HIV) and HCV co-infection are at increased risk of progression to cirrhosis compared with persons with HCV alone, but the reasons for this are unclear. Up to 80% of HIV+ iv drug users and 98% of HIV+ haemophiliac cohorts are co-infected with HCV (Mohsen et al., 2002).

In co-infected individuals, the HCV-specific CD8 response, but not that to HIV and EBV, has been shown to be strongly dependent on absolute CD4 count (Kim et al., 2005), indicating a particular requirement for CD4 “help” in maintaining responses to HCV.

To investigate whether co-infection with HIV directly influences HCV-specific CD4 responses, analysis of IFN- $\gamma$ -secreting CD4<sup>+</sup> responses to HCV antigens in a cohort of HCV mono-infected and HCV/HIV co-infected children with haemophilia revealed a major decrease in HCV-specific responses among those who were co-infected (Harcourt et al., 2006).

To assess the proliferative capacity of CD4 cells, a group of HCV/HIV co-infected and a group of HCV mono-infected individuals were tested for responses to HIV and HCV recombinant proteins (Lauer et al., 2002). In the HCV mono-infected group 47% had proliferative responses to HCV, whereas no HCV responses were detectable in any of the co-infected individuals.

Furthermore, responses of intrahepatic CD4<sup>+</sup> T cells in co-infected individuals show lower IL-10 secretion when compared with HCV mono-infected individuals which may have implications for HCV-related disease progression (Graham et al., 2004).

Overall, these data suggest that the loss of HCV-specific CD4 cells occurs readily during HIV co-infection and additionally a change in functionality may occur; these modifications have a profound influence on cellular immunity against HCV.

## **6. Viral escape from immune responses**

### **6.1. CD4<sup>+</sup> T cell escape and viral persistence**

Numerous studies in both animal and human models have documented immune escape from virus-specific CTL responses by viral mutations in CTL epitopes that lead to loss of immune control and viral persistence (Allen et al., 2000; Altfeld et al., 2002; Borrow et al., 1997; Erickson et al., 2001; Goulder et al., 1997; O'Connor et al., 2002; Vogel et al., 2002).

Less information is currently available about the potential for immune escape from viral CD4<sup>+</sup> T cell epitopes, although limited studies in chronic HIV and HCV infection have identified multiple autologous virus variants for specific CD4<sup>+</sup> T cell epitopes (Eckels et al., 1999; Harcourt et al., 1998; Wang and Eckels, 1999). Peptides corresponding to viral variants were synthesised and tested in *in vitro* assays, and the majority of variants failed to stimulate proliferation or cytokine production by CD4<sup>+</sup> T cells (Eckels et al., 1999; Harcourt et al., 1998; Wang and Eckels, 1999). These data suggest that viral immune escape from specific CD4<sup>+</sup> T cell responses is possible, although the consequences of CD4<sup>+</sup> immune escape on viral persistence are currently unclear and await further investigation.

That escape mutants may play a role in HCV persistence has also been shown previously in a study with 4 HLA-DRB1\*15 patients chronically infected with HCV (Wang et al., 2003).

Here, naturally occurring single amino acid substitutions in the DRB1\*15-restricted Th1 epitope (aa 358-375) in the NS3 protein failed to stimulate proliferation. This was also accompanied by a shift in cytokine secretion patterns from one characteristic of a Th1 anti-viral response to a Th2 form.

In addition to escape from virus-specific T cell responses, escape from neutralising antibody (nAb) responses is thought to be one potential mechanism leading to the persistence of some viruses (Ciurea et al., 2000; Parren et al., 1999). Recent data generated in an LCMV model have provided additional insight into the relationship between CD4<sup>+</sup> T cells and immune escape from nAb responses. CD8<sup>-/-</sup> mice were infected with the WE strain of LCMV to establish a long-term infection with high levels of virus production that is transiently controlled by nAbs (Ciurea et al., 2000).



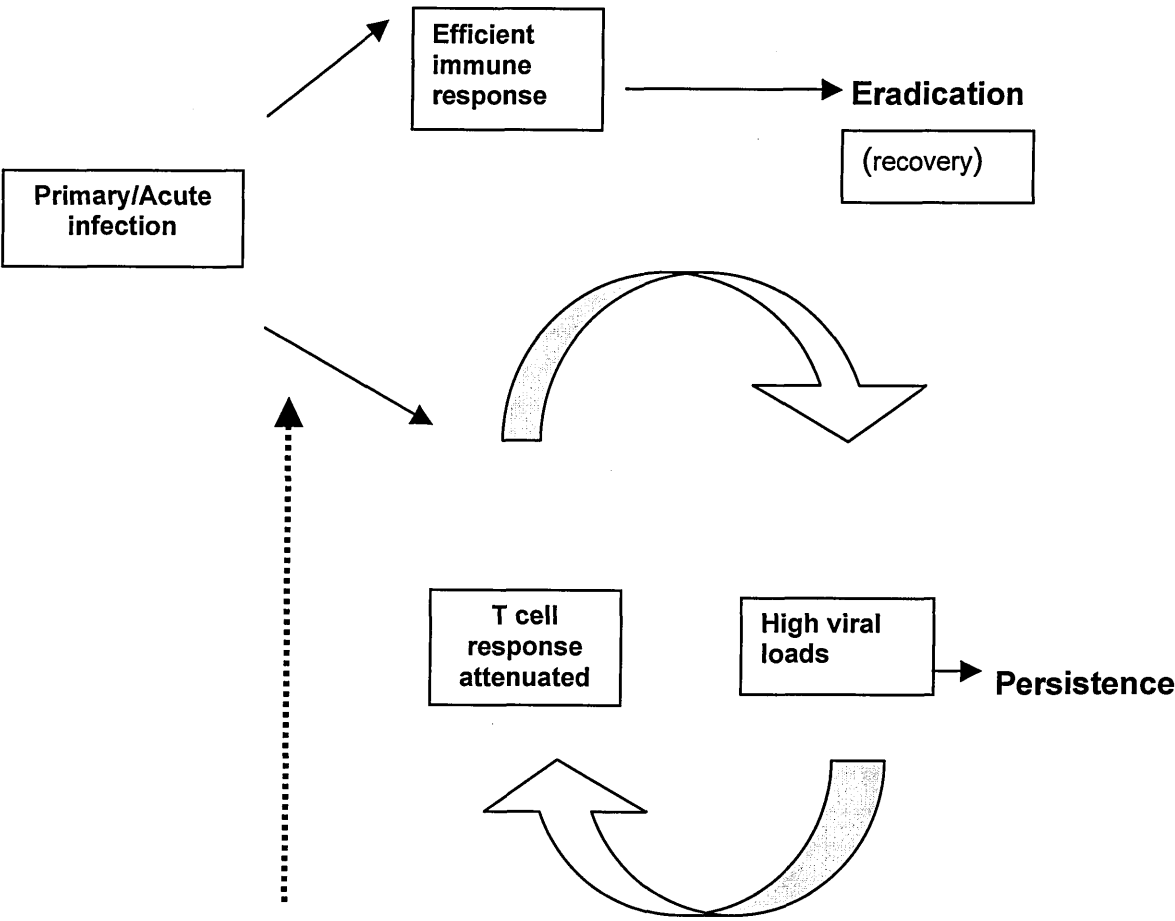
However, the lack of CD8<sup>+</sup> CTL responses and consequently high viraemia in this model leads to escape from polyclonal nAb responses as well as rapid induction of CD4<sup>+</sup> T cell unresponsiveness (Ciurea et al., 2001). Although the molecular mechanism of CD4<sup>+</sup> T cell unresponsiveness is not clear from this study, it has been postulated that the high antigenic load in this model system may have resulted in activation of all virus-specific CD4<sup>+</sup> T cells, leading to exhaustion and activation-induced cell death as has been described for CTLs (Gallimore et al., 1998; Moskophidis et al., 1993b). Nevertheless, in the absence of LCMV-specific CD4<sup>+</sup> T cells, these mice failed to generate new effective humoral responses against emerging neutralisation-escape mutants and the viral infection persisted (Ciurea et al., 2001). These data provide further evidence for the importance of interactions between the cellular and humoral immune responses for efficient control of viral infections.

Recent years have seen a revival of interest in the role of regulatory T cells – notably the CD4<sup>+</sup> CD25<sup>+</sup> subset. It is plausible that in HCV infection excessive regulation is involved in the suppression of HCV specific T-cell responses.

Recently, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cell activity has been shown to be present in patients with chronic HCV infection, which may contribute to weak HCV-specific T-cell responses and viral persistence (Boettler et al., 2005; Cabrera et al., 2004; Rushbrook et al., 2005; Sugimoto et al., 2003).

An important question, which derives from these studies, is to what extent the Treg activity seen in persistent infection relates to the activity of antigen-specific cells. T cells, which are repetitively stimulated with antigen over time may develop regulating characteristics. Such cells might downregulate both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in persistent infection, particularly within the inflamed liver.

Other mechanisms leading to HCV viral escape have mainly been shown for CD8<sup>+</sup> T cells. I therefore have summarised these mechanisms in figure 1.3.



**Potential mechanisms that attenuate T-cell responses during HCV infection leading to “T cell failure”**

**References**

<b>T cell exhaustion</b>	(Gallimore et al., 1998; Moskophidis et al., 1993a)
<b>Viral variation leading to ...</b>	
○ Viral escape	(Kaneko et al., 1997; Timm et al., 2004; Tsai et al., 1998)
○ T cell antagonism	(Bertoletti et al., 1994)
○ “Original antigenic sin”	(Klenerman and Zinkernagel, 1998)
<b>T cell dysfunction</b>	
○ Stunning	(Lechner et al., 2000)
○ Stunting	(Appay et al. 2002)
○ Viral modulation	(Yao et al. 2001)
○ Liver modulation	(Bertolino et al. 1998)
○ Abnormal regulation (Treg cells)	(Rushbrook et al. 2005, Boettler et al. 2005)
<b>Failure of other immune cells</b>	
○ B cells	(Farci et al. 1992; Farci et al.1994; Farci et al. 1996)
○ Innate cells	(Crotta et al. 2002;Tseng et al. 2002)

**Figure 1.3. A simple model for T cell failure in HCV infection.**

During primary acute infection, an efficient immune response will lead to viral control and/or eradication. A variety of mechanisms may subvert this initial response. Once high viral loads are established T cell exhaustion in conjunction with a variety of other mechanisms may lead to further attenuation of T cell responses and viral persistence.

**7. Aim of the thesis**

HCV infection represents a major global health problem. Although 20 to 50% of infected people are able to spontaneously resolve HCV infection, the determinants of viral clearance following acute HCV infection versus establishment of chronic HCV infection are not clearly defined and it may be necessary to use a variety of techniques in the detection of the functional status. Increasing evidence indicates that induction of a strong cellular immune response involving both cytotoxic T lymphocytes (CTL) and CD4<sup>+</sup> T helper cells is essential for spontaneous control of chronic viral infections.

The aim of my thesis was to assess the role of HCV-specific CD4<sup>+</sup> T cell responses in a setting of spontaneously resolved and chronically infected individuals as well as in HCV-exposed individuals with antibody deficiency. Possible reasons for persistent infection could be a dysfunction of virus-specific T cells. Using different techniques I was able to analyse cytokine and proliferative profiles in these patients.

Knowledge about the frequency and function of HCV-specific CD4<sup>+</sup> T cell responses and their kinetics is of great importance and therefore not only will help to understand the mechanisms behind the failure of HCV clearance but also will contribute to the establishment of new immunotherapeutic strategies.

## **CHAPTER 2**

### **MATERIALS AND METHODS**

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## 1. Ethics

Signed informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Approval for this study was formally obtained from the ethics committees at the John Radcliffe Hospital, Oxford, the Royal Free Hospital, London, and the South Birmingham local research ethics committee.

## 2. Clinical definitions

### *(1) Chronic HCV (RNA+)*

Chronic (or persistent) HCV infection is defined as the detection of HCV RNA by PCR (detection limit of 300 HCV RNA copies/ml of plasma; v2.0 Amplicor assay, Roche Diagnostics Ltd, Basel, Switzerland), on at least two consecutive occasions 6 months apart.

### *(2) Spontaneously resolved HCV (RNA-)*

Spontaneously resolved HCV infection is defined as the absence of detectable HCV RNA by PCR (v2.0 Amplicor assay, Roche Diagnostics Ltd), in the presence of HCV antibodies (Abbott HCV EIA 3.0, Abbott Laboratories, Abbott Park, Illinois) on at least two consecutive occasions 6 months apart.

### 3. Peripheral blood samples

Peripheral blood samples were obtained from patients with resolved or persistent HCV infection, usually by clinic staff at the John Radcliffe hospital, Oxford and sometimes by myself. Samples were collected in 50 ml Falcon tubes or vacutainers (Becton Dickinson) containing sodium heparin. Blood samples from healthy donors were obtained from work colleagues. Samples were transported within 4 hours to the laboratory for isolation of peripheral blood mononuclear cells (PBMC) for further analysis.

PBMCs were isolated by density gradient centrifugation. Briefly whole blood was gently layered onto Lymphoprep (Nycomed, Oslo, Norway), at a ratio of 1.4 ml blood: 1 ml Lymphoprep. This was centrifuged at 2,200 rpm for 25-30 minutes with no brake. The PBMC fraction was removed and washed once at 1800 rpm for 10 minutes and again at 1200 for 7 minutes. Viable cells were enumerated by trypan blue exclusion. PBMCs were then resuspended in appropriate medium or immediately frozen for further analysis.

As for the RIBA-indeterminate patients (chapter 3) the PBMC were obtained already cryostored from the Blood Transfusion Service in Birmingham. Compared to fresh PBMC some of the antigen-presenting cells in cryostored PBMC might have reduced viability leading to different or reduced responses. Unfortunately, this limitation in a part of my studies was not avoidable.

#### 4. Freezing and thawing of PBMC

PBMC were pelleted and then resuspended in 0.5 ml of FCS on ice. 0.5 ml of freshly prepared freezing media (freezing media; 9:1 ratio of FCS:DMSO) was cooled on ice and added to the cells in FCS, which were resuspended and transferred immediately into cold freezing vials (Nunc, U.S.A).

Where possible PBMCs were frozen in aliquots of 10-20x 10<sup>6</sup>/vial and stored in liquid nitrogen. To defrost frozen PBMC, vials of cells were rapidly thawed in a water bath and immediately washed in RPMI before further use.

#### 5. Proliferation assays

##### *[<sup>3</sup>H] thymidine assays*

Proliferation assays were performed using fresh PBMC, cultured in 96-well round bottomed microtiter plates at a concentration of 2 x 10<sup>5</sup>/well in triplicate. Cells were cultured in RPMI 1640 (Life Technologies) with 10% heat inactivated human AB serum at 37°C for 5 days with 5% CO<sub>2</sub> and 100% humidity in the presence of HCV proteins or peptides and CMV-Lysate in selected cases. Peptides were added at a concentration of 10 µg/ml and proteins at 1 µg/ml. The proliferative response was evaluated following 18 hours incubation with [<sup>3</sup>H] thymidine, before harvesting and counting in a Topcount scintillation counter (Packard, Canberra, Australia). Positive control antigens were used in each proliferation assay and included PHA as a non-specific stimulator, and tetanus toxoid (2µg/ml) as a recall antigen.



As recombinant test proteins were produced using *E. coli* and Yeast expression systems, proliferative responses against control Yeast and *E. coli* antigens were also measured. Responses for these latter antigens were always negative.

Results are expressed as counts per minute (cpm) using a scintillation counter, or as stimulation indices (SI), i.e. the geometric mean of the test cultures' cpm divided by the geometric mean of the background cultures' cpm, where a response is considered positive if the SI was greater than 3.

#### *CFSE-based proliferation assays*

For staining with CFSE (Molecular Probes, Eugene, Oregon, USA) PBMC at  $1 \times 10^7$  /ml in PBS were incubated at 37°C for 7 min with 0.5 $\mu$ M CFSE. CFSE labelling was terminated by washing the cells with PBS containing 10% pooled human serum and then with PBS only. The cells were then resuspended at  $2 \times 10^6$ /ml in RPMI containing 10% human serum. Stained cells ( $1 \times 10^6$ /well, 1ml) were cultured in 48-well plates with medium alone, PHA as a positive control, core peptide pools 1-4 (10 $\mu$ g/ml final concentration) and non-structural proteins NS3-5 (1 $\mu$ g/ml final concentration). A recombinant yeast control protein (1 $\mu$ g/ml final concentration; Chiron, Emeryville, CA, USA) and a lysate of CMV- infected cells (CMV-Lysate; optimized final concentration 0.05 $\mu$ g/ml; Virusys, North Berwick, ME, USA) were also included as controls.

After six days of culture, cells for each antigen were transferred into FACS tubes, washed in PBS and stained at 4°C with the following antibodies: anti-human CD4-APC, CD8-PE and Viaprobe (7-AAD) to exclude dead cells (all BD Pharmingen, Oxford, UK).

Flow cytometric analysis was performed on a FACSCalibur and analysis performed using CellQuest software (BD Biosciences) and/or FlowJo (Treestar, Ashland, Oregon, USA).

The number of cells that had proliferated was determined by gating on the lineage-positive CFSE<sup>low</sup> subset. After normalisation for the cell input number the stimulation index (SI) was calculated using the following formula:

Number of CD4<sup>+</sup> CFSE<sup>low</sup> cells with antigen

Number of CD4<sup>+</sup> CFSE<sup>low</sup> cells without antigen

A SI > 2 was considered to represent a positive response, as previously defined (Mannering et al., 2003).

The CD4<sup>+</sup> proliferative frequency (%) was calculated only for those where the SI was positive, using the following formula:

Number of CD4<sup>+</sup> CFSE<sup>low</sup> cells

(Number of CD4<sup>+</sup> CFSE<sup>low</sup> cells + number of CD4<sup>+</sup> CFSE<sup>high</sup> cells) x100

The final proliferative frequency (%) was achieved by subtracting the proliferative frequency with antigen from the proliferative frequency without antigen.

*Calculation of cell divisions and precursor frequency:* Stimulation of cells with PHA induces polyclonal stimulation of T cells resulting in cell division with distinct CFSE fluorescence peaks, allowing determination of the mean CFSE fluorescence for each generation. These values were used to calculate the average number of cell divisions in cells stimulated with antigen. Precursor frequency was estimated by dividing the number of CFSE<sup>low</sup>/CD4<sup>+</sup> populations by  $2^x$ , where x is the average number of cell divisions, to determine the absolute number of precursors for the CFSE<sup>low</sup>/CD4<sup>+</sup> cells, and then dividing this value by the total number of cells analysed, as previously described (Lyons, 2000; Novak et al., 1999b).

Division index and proliferation index were calculated using FlowJo software. The division index is the average number of divisions that a cell (that was present in the starting population) has undergone.

For example, if half of the cells in the starting population divided and the average number of divisions was 4, the Division index would be 2.

The proliferation index is the average number of divisions that those cells, *which divided* underwent. For example, if the average number of divisions for all the CFSE<sup>low</sup> cells was 4, the Proliferation index would be 4. These statistics are related in the following way:

Division Index= (Proliferation Index x Precursor-frequency)/100

## 6. ELISpot assays

ELISpot assays were performed using fresh or frozen PBMC. Multiscreen filtration plates (MAIP 54510, Millipore UK Ltd) were coated with 50µl monoclonal antibody (mAb) to IFN- $\gamma$  (Mabtech) diluted to 15µg/ml with sterile-filtered phosphate buffer saline (PBS), and left overnight at 4°C. Plates were washed with 6x 200 µl PBS before blocking with 200µl RPMI 1640 (Life Technologies) plus 10% heat-inactivated FCS (Sigma Aldrich) and incubated for 3 hours at 37°C. PBMC's were added to the plate at a concentration of  $2 \times 10^5$ /well. Protein or peptide was added to each well in duplicate. Peptides were added at a concentration of 10 µg/ml and proteins at a concentration of 1µg/ml. Plates were incubated for 18 hours overnight at 37°C with 5% CO<sub>2</sub> and 100% humidity. Plates were then washed with 6x 200µl sterile filtered PBS before addition of 50µl biotinylated mAb (Mabtech) diluted 1 in 1000 in sterile filtered PBS and incubated for 2 hours at room temperature.

Plates were washed with 6 x 200µl sterile filtered PBS before addition of 50µl streptavidin-alkaline phosphatase (Mabtech) diluted to 1:1000 in sterile filtered PBS and incubation for 1 hour at room temperature. Finally, plates were washed with 6x 200µl sterile filtered PBS, and 50µl substrate (BCIP/NBT Biorad) was added and incubated until dark spots appeared. Colour development was stopped by washing in tap water (3x200 µl/well). The plates were left to dry and spots were counted on an ELISpot reader (AID, Strassberg, Germany). There is currently no consensus on the optimal way to identify and enumerate positive responses in an ELISpot assay.

I have employed one of two techniques; a test is considered positive if the probability of a spot appearing in the test well is significantly different ( $p < 0.05$ ) from the probability of a spot appearing in the control well, assuming a Poisson distribution. Quantification is then determined by subtracting the mean background number of spots from the mean number of spots in the test well. Alternatively, a positive response may be defined using an arbitrary cut off such that the mean number of spots per test wells minus the mean number of spots in the background wells exceeds that cut-off. One of these two methods was used consistently in each particular study.

### **6.1. IL-2/IFN- $\gamma$ ELISpot (for chapter 4 and 5)**

PBMC were tested in both interferon- $\gamma$  (IFN- $\gamma$ ) (MABTECH, Stockholm, Sweden) and Interleukin-2 (IL-2) (BD Biosciences, Oxford, UK) ELISpot assays as per manufacturer's instructions. According to the manufacturer's reference, reproducibility of the IL-2 assay appears similar to that of the IFN- $\gamma$  ELISpot, and the sensitivity of this assay lends itself to ex vivo measurement of even very low frequencies of cytokine-producing cells (potentially down to 1/300,000).

Briefly, whole or CD8-depleted PBMCs (0.2 million/well) were plated in 96-well ELISpot plates precoated with anti-IFN- $\gamma$  or anti-IL-2. Antigens used for analyses were HCV Core-derived peptides (20mers overlapping by 10) spanning aa 1-191 arranged into 3 pools of 5 peptides and 1 pool of 3 peptides (10 $\mu$ g/ml final concentration for each peptide; pool 1, 2, 3, 4). The recombinant proteins NS3, NS3/4, NS4, and NS5 (Chiron, Emeryville, CA, USA) were used at a final concentration of 1  $\mu$ g/ml as previously described (Barnes et al., 2002).

Medium alone was used as a negative control and phytohaemagglutinin (PHA) was used as a positive control for all assays. Tetanus toxoid (TT) (2 µg/ml, Evans Vaccines, Liverpool UK) and cytomegalovirus (CMV) lysate (4 µg/ml, Virusys, North Berwick, ME, USA) were also included in all assays. The plates were developed after 18-20 hours using AEC Chromogen color reagent for IL-2 (BD Biosciences, Oxford, UK) and AP color reagent for IFN-γ (BioRad, CA, USA) analysed for spot forming cells (SFCs) using an ELISpot plate reader (AID Reader System, Strassberg, Germany, ELISpot 3.1 SR program). Assays with high background (average 10 SFC/well in negative control wells) or no PHA response were excluded. The frequency of IFN-γ<sup>+</sup> or IL-2<sup>+</sup> T cells specific for each antigen was calculated by subtracting the average SFC in negative control duplicate wells from the average SFC in stimulated duplicate wells and expressed as HCV-specific IFN-γ or IL-2 SFCs/10<sup>6</sup> PBMCs.

A positive response required wells with SFCs/well greater than 3 SD above mean negative control response or that the probability of a spot appearing in the stimulated well was significantly different ( $p < 0.05$ ) from the probability of a spot appearing in the negative control well, assuming a Poisson distribution (Excel BINOMDIST statistics program; Microsoft) (Barnes et al., 2002). The mean background in the negative control wells was 5 spots/200,000 PBMC for IFN-γ (SD 1.8) and 3 spots/200,000 PBMC for IL-2 (SD 2.1).

**6.2. IFN- $\gamma$  Matrix ELISpot (chapter 6)**

For analyses, we used 301 20 amino acid long peptides overlapping by 10 amino acids arranged into 58 pools spanning the whole HCV genome to detect T cell responses in individual patients independent of HLA type. Additionally specific HLA-A2- and HLA-A1-restricted peptides were tested individually according to the subjects' tissue types. These were HCV-NS3 peptide HLA-A2-CINGVCWTV, EBV HLA-A2-GLCTLVAML and HCV HLA-A1-ATDALMTGY. Further antigens were tetanus Toxoid (TT) and CMV-lysate (CMV-L). Control wells included medium alone and PHA.

## 7. Peptides and antigens used in assays

Genotype 1a antigens used for analyses were core-derived peptides (20mers overlapping by 10) arranged into 3 pools of 5 peptides each and one pool of 3 (10µg/ml final concentration, Pool 1-4) (Table 2.1). The rest of the genome was spanned using recombinant proteins NS3 (aa 1192-1457), NS3/4 (aa 1192-1931), NS4 (aa 1569-1931) and NS5 (aa 2054-2995;)(final concentration 1µg/ml [Chiron, USA]) as previously described. The difference of the concentrations in molarity is 4.5µM for the core peptide pools 1-4, 36nM for NS3, 26nM for NS4 and 9.7nM for NS5. Control wells included yeast protein, medium alone and PHA.

C-1	1-20	MSTNPKPQRKTKRNTNRRPQ
C-2	11-30	TKRNTNRRPQDVKFPGGGQI
C-3	21-40	DVKFPGGGQIVGGVYLLPRR
C-4	31-50	VGGVYLLPRRRGPRLGVRATR
C-5	41-60	GPRLGVRATRKTSESRQPRG
C-6	51-70	KTSESRQPRRRQPIPKARR
C-7	61-80	RRQPIPKARRPEGRTWAQPG
C-8	71-90	PEGRTWAQPGYPWPLYGNEG
C-9	81-100	YPWPLYGNEGCGWAGWLLSP
C-10	91-110	CGWAGWLLSPRGSRPSWGPT
C-11	101-120	RGSRPSWGPTDPRRRSRNLG
C-12	111-130	DPRRRSRNLGKVIDTLRCGF
C-13	121-140	KVIDTLTGCFADLMGYIPLV
C-14	131-150	ADLMGYIPLVGAPLGGAARA
C-15	141-160	GAPLGGAARALAHGRVLED
C-16	151-170	LAHGVRVLEDGVNYATGNLP
C-17	161-180	GVNYATGNLPGCSFIFLLA
C-18	171-190	GCSFSIFLLALLSCLTVPAS

**Table 2.1. HCV core overlapping peptides used in ELISpot and other assays**



## 8. Modulation of function by addition of cytokines

For cytokine modulation, human recombinant cytokine IL-2 (Proleukin, Chiron) was added at 100 U/ml at the beginning of the ELISpot assay, while IL-15 (Peprotech, Rocky Hill, NJ, USA) was added at 100 ng/ml to the PBMC with or without antigen. The doses of cytokines were selected based on published dose-response experiments in which SFC responses were observed to peak at 100 U/ml for IL-2 and 100ng/ml for IL-15 (Chitnis, 2003). These doses for IL-2 and IL-15 have been reported as being optimal for preventing T-cell apoptosis.

## 9. IFN- $\gamma$ /IL-2 secretion assay

$10 \times 10^6$  PBMC were stimulated at 37°C with 10 $\mu$ g/ml HCV peptides or 1 $\mu$ g/ml proteins as used in the ELISpot for 6 h or 16 h, respectively. IFN- $\gamma$  or IL-2 secreting cells were isolated using a human IFN- $\gamma$  or IL-2 secretion assay (Miltenyi Biotec, Bergisch Gladbach, Germany) as per manufacturer's instructions. The principle of this assay is as follows: Antigen-specific T cells are analysed and isolated using this assay by restimulating PBMCs for a short period of time with specific peptide or protein. Subsequently, an IFN- $\gamma$ - and IL-2-specific **Catch Reagent** is attached to the cell surface of all leucocytes. The cells are then incubated for a short time at 37°C to allow cytokine secretion. The secreted IFN- $\gamma$  and IL-2 bind to the IFN- $\gamma$ - and IL-2-specific Catch Reagent on the positive, secreting cells.

These cells are subsequently labelled with a second IFN- $\gamma$ - and IL-2-specific antibody, the **IFN- $\gamma$  and IL-2 Detection Antibody** conjugated to PE and APC, respectively, for sensitive detection by flow cytometry. The cytokine secreting cells can then be magnetically labelled with **Anti-PE and Anti-APC MicroBeads** and enriched over a MACS column, which is placed in the magnetic field of a MACS separator. The magnetically labelled cells are retained in the MACS column while unlabelled cells run through. After the column has been removed from the magnetic field, the magnetically retained cells can be eluted as positively selected cell fraction, enriched for cytokine secreting cells.

Cells are further stained with CD14-PerCP, CD19-PerCP and ViaProbe (in order to exclude monocytes, B cells and dead cells, all of which may bind non-specifically to the PE/APC magnetic beads) (all BD Pharmingen, San Diego, CA) and CD4-FITC (Miltenyi Biotec) and incubated on ice for 20 min.

Anti-PE and anti-APC microbeads were used to enrich PE- (IL-2) and APC- (IFN- $\gamma$ ) stained cells by two rounds on magnetic separation columns (MS+ columns, Mini MACS, Miltenyi Biotec).

To calculate the percentage of cytokine<sup>+</sup>/CD4<sup>+</sup> cells, where cytokine-positive cells were not detectable pre-enrichment, we used the following formula: absolute number of cytokine-positive cells in the post-enrichment sample/(total number of CD4<sup>+</sup> T-cells in the pre-enrichment sample) x 9. The factor 9 is due to the acquisition of 10% of the stained sample as the “pre-enrichment” sample with the remainder (90%) of the cells being the source of the “post-enrichment” sample.

This formula has been previously adopted for the calculation of the percentage of cytokine-secreting or tetramer positive cells following magnetic enrichment (Miltenyi Biotec) (Barnes et al., 2004b; Day et al., 2003).

## 10. CD8 and CD25 Depletion

CD8<sup>+</sup> and CD25<sup>+</sup> cell depletions were performed using magnetic bead separation (Dynal, Oslo, Norway) as per manufacturer's instructions. CD8<sup>+</sup> T cell depletion was efficient at removing 99% of CD8<sup>+</sup> T cells as determined by flow cytometry. Because CD8 depletion resulted in relative CD4 enrichment, calculation of HCV-specific CD4 T-cell frequency in CD8-depleted PBMCs included the CD4 enrichment factor (%CD4 in CD8-depleted PBMCs / %CD4 in total PBMCs) as determined by flow cytometry. For example, with 2-fold CD4 enrichment following CD8 depletion (e.g., 15% CD4 in whole PBMCs, 30% CD4 in CD8-depleted PBMCs), IFN- $\gamma$ <sup>+</sup> or IL-2<sup>+</sup> SFCs in 200,000 CD8-depleted PBMCs/well were divided by 2 to compensate for the 2-fold CD4 enrichment in each well (compared with 200,000 whole PBMCs/well). Thus, for 50 IFN- $\gamma$ <sup>+</sup> or IL-2<sup>+</sup> SFCs/200,000 CD8-depleted PBMCs/well, the result was calculated as (50 IFN- $\gamma$ <sup>+</sup> or IL-2<sup>+</sup> SFCs/0.2 million PBMCs per well)/2 = 125 IFN- $\gamma$ <sup>+</sup> or IL-2<sup>+</sup> SFCs/million PBMCs.

## 11. CD62L Depletion

CD62L (Miltenyi Biotec, Bergisch Gladbach, Germany) cell depletions were performed using magnetic bead separation as per manufacturer's instructions. CD62L T cell depletion was efficient at removing 95% of CD62L<sup>+</sup> T cells as determined by flow cytometry. Because CD62L depletion resulted in relative CD4 enrichment, calculation of HCV-specific CD4<sup>+</sup> T-cell frequency in CD62L-depleted PBMCs included the CD4 enrichment factor (%CD4 in CD62L-depleted PBMCs / %CD4 in undepleted PBMCs) as determined by flow cytometry.

For example, with 1.5-fold CD4 enrichment following CD62L depletion (e.g., 30% CD4 in undepleted PBMCs, 45% CD4 in CD62L-depleted PBMCs), IFN- $\gamma$ <sup>+</sup> SFCs in 200,000 CD62L-depleted PBMCs/well were divided by 1.5 to compensate for the 1.5-fold CD4 enrichment in each well (compared with 200,000 whole PBMCs/well). Thus, for 50 IFN- $\gamma$ <sup>+</sup> SFCs/200,000 CD62L-depleted PBMCs/well, the result was calculated as (50 IFN- $\gamma$ <sup>+</sup> SFCs/0.2 million PBMCs per well)/1.5 = 167 IFN- $\gamma$ <sup>+</sup> SFCs/million PBMCs.

## 12. Tetramer staining (chapter 6)

HLA class I-peptide tetramers were prepared and staining was performed as previously described (Lechner et al., 2000; Lucas et al., 2004), and included tetramers specific for an epitope restricted by HLA-A2 (HCV-NS3 peptide 1073-1081, CINGVCWTV).

Briefly,  $1 \times 10^6$  PBMC were pelleted in a FACS tube and the supernatant was removed by blotting on paper. 0.5-1.5  $\mu$ l of tetramer was added to the pellet, which was then incubated at 37°C/ 5% CO<sub>2</sub>/100% humidity for 25 minutes. Cells were washed in PBS/1% FCS and then stained with anti-CD8 Ab-FITC/-PerCP at 4°C for 20 minutes (in addition to other antibodies of interest). Cells were washed in PBS/1% FCS and then fixed with 1% formaldehyde before FACS analysis.

13. Flow cytometry

Flow cytometric analysis was performed on a FACSCalibur, and analysis was performed using CellQuest software (BD Biosciences). Antibodies used are shown below:

Antibody	Manufacturer
Anti-CD8	BD Pharmingen
Anti-CD4a	BD Pharmingen
Anti-CD4b	Miltenyi Biotec
Anti-CD14	BD Pharmingen
Anti-CD19	BD Pharmingen
Anti-CD25	BD Pharmingen
Anti-CD62L	BD Pharmingen
ViaProbe (7-AAD)	BD Pharmingen

## 14. Statistical analysis

Pooled data are presented as the mean. The Mann-Whitney and Fisher's exact statistical tests, the paired *t* test and the linear regression analysis were performed using Prism V3 software (Graphpad, San Diego, CA). A P value of 0.05 or less was considered statistically significant. Additionally, the binomial exact method in Fig. 3.1. of chapter 3 was performed using Stata version 7 software (StataCorp., TX, USA).

## CHAPTER 3

# T CELL RESPONSES AND PREVIOUS EXPOSURE TO HEPATITIS C VIRUS IN RIBA INDETERMINATE BLOOD DONORS

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## 1. Abstract

**Background:** Some individuals have weak or restricted virus-specific antibodies to HCV, and are classified as “indeterminate” on RIBA testing. Such donors are almost always negative for viral RNA in blood. I postulated that previous transient virus exposure might account for some of these cases.

**Methods and findings:** With sensitive ex-vivo analyses of T cell responses, I identified virus-specific responses in 15 of 30 indeterminate blood donors tested, compared with none in controls ( $p=0.0013$ ). Additionally, these responses were typically focused on core-derived peptides. These findings suggest previous exposure to the virus in many blood donors with indeterminate serology.



## 2. Introduction

Hepatitis C virus (HCV) is a major cause of liver disease worldwide and the screening of blood products is a crucial part of its prevention (Lauer and Walker, 2001). Screening tests for this infection are based on enzyme immunoassays (EIA) for anti-viral antibodies. EIA results might subsequently be confirmed by a specific recombinant immunoblot assay (RIBA). In this assay, unlike the initial EIA, antibodies to four individual viral antigens are immobilised on a nitrocellulose strip and tested: a positive result is defined as response to two or more antigens (Fabrizi et al., 2001). After infection, the virus typically becomes persistent, and chronic virus carriers remain positive by antibody testing (EIA and RIBA) and positive for viral RNA in the blood. 15–20% of individuals clear the virus spontaneously and remain negative for viral RNA in the long term (Lauer and Walker, 2001; Takaki et al., 2000). Such spontaneous resolvers include rare individuals in whom acute HCV infection is seen clinically, but they usually present without a history of acute infection. These spontaneous resolvers show positive antibody test results in the absence of detectable circulating virus. Another group of blood donors have indeterminate results: ie they have RIBA test results that are weak or narrowly focused. Such donors represent 193 (0.012%) of 1,576,197 over 8 years at the National Blood Service Birmingham centre (Surveillance, January 2003). This proportion compares with 0.06% of all donors in the UK (0.18% of new donors) who tested positive for anti-viral antibodies over this period (Surveillance, January 2003)

In other settings such as high-risk groups, individuals with indeterminate RIBA results could represent about 2% of all positive tests, or 12% of those who test weakly positive by EIA (Dufour et al., 2003).

So far, whether such individuals with indeterminate results have non-specific antibodies that are cross-reactive to viral antigens but unrelated to the infection, or whether their responses represent a variant response to the virus, is unclear. Since (indeterminate) donors usually give negative results in PCR testing, such tests cannot be used to distinguish between these two possibilities (Dufour et al., 2003). Although antibody reactivity declines over time after spontaneous resolution of infection, T cell responses might be maintained as has been previously reported (Takaki et al., 2000). I have studied T cell responses with enzyme-linked immunospot assays (ELISpot) (Barnes et al., 2002) for detection of interferon- $\gamma$ - secreting cells in a group of indeterminate blood donors and compared them with those seen in typical spontaneous resolvers of HCV.

### 3. Results

#### 3.1. Study subjects

T cell responses by ELISpot in a group of “indeterminate” blood donors were studied and compared to those found in typical spontaneous resolvers (SR) of HCV infection (recruited from the local hepatology outpatient department). 14 healthy individuals from my lab were also included as a control group (8 females, 6 males, age range 23-42).

“Indeterminate” donors (EIA+/RNA-/RIBA-indeterminate) were identified through standard blood screening procedures at the National Blood Service Birmingham Centre (19 males, 11 females; median age 46 years, range 31-68 years; table 3.1). PBMC were obtained and tested in interferon- $\gamma$  ELISpot assays as previously described (chapter 2).

**Table 3.1. Baseline characteristics of RIBA “indeterminate” blood donors**

C33=NS3; c22=core;

Subject	Age	Sex	1. RIBA test	RIBA Follow-up
1	60	F	c33	c33
2	40	F	c33	c33
3	55	F	NS5	NS5
4	45	F	c33	No follow-up
5	42	F	c33	c33
6	56	F	c33	c33
7	31	F	c33	c33
8	65	F	NS5	NS5
9	56	F	NS5	NS5
10	41	F	NS5	NS5
11	52	F	c33	c33, NS5
12	62	M	c33	c33
13	52	M	c22	c22
14	74	M	c33	c33
15	56	M	c33	c33
16	51	M	c33	c33
17	56	M	c33	c33
18	54	M	NS5	NS5
19	49	M	c33	c33
20	65	M	c33	Neg
21	43	M	c33	c33
22	54	M	c33	c33
23	68	M	c33	c33
24	45	M	c33	c33
25	45	M	NS5	NS5
26	43	M	c33	c33
27	47	M	NS5	NS5
28	43	M	c33	c33
29	50	M	c33	c33
30	66	M	c33	c33

Table 3.2. Raw data responses of RIBA-indeterminate donors in ELISpot

Subject	P1	P2	P3	P4	NS3	NS3/4	NS4	NS5
1	0	110	0	0	0	0	0	0
2	0	1373	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	365	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	0	0	98	70	0	0	33	30
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	82,5	0	105	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	60	58	52	0	58	0	0
16	0	0	0	0	0	0	0	0
17	0	668	0	0	0	0	0	0
18	0	513	0	0	0	0	0	0
19	0	65	0	0	0	0	0	0
20	0	342	0	0	0	0	0	0
21	0	143	0	0	0	0	0	0
22	0	715	0	0	0	0	0	0
23	48	447	0	0	0	0	0	0
24	0	148	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0
28	0	75	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0

Responses are shown as SFC/10<sup>6</sup> PBMC

**Table 3.3. Raw data responses of spontaneous resolvers in ELISpot**

Subject	P1	P2	P3	P4	NS3	NS3/4	NS4	NS5
R1	80	0	0	0	63	33	0	25
R2	55	55	118	35	95	60	0	0
R3	0	93	28	0	0	100	55	0
R4	138	200	178	0	160	0	0	0
R5	0	0	0	0	0	0	0	0
R6	0	168	80	0	0	193	65	0

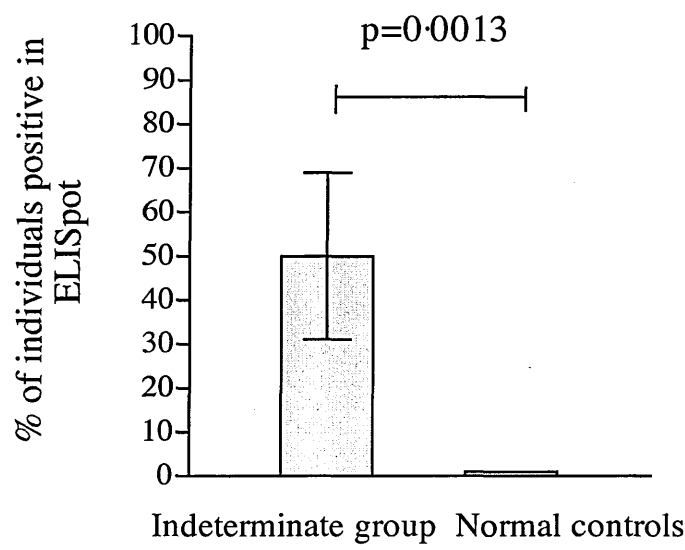
P1, P2, P3, P4= pools of peptides spanning the whole HCV core region; NS3-5= recombinant HCV non-structural proteins

**3.2. RIBA-indeterminate results**

22 blood donors had RIBA reactivity against band c33c (NS3), one against c22 (core), and seven against NS5. 29 of the 30 donors were followed up for a median of 30 months (IQR 9–36.5 months); after repeated tests over this period, 27 of these 29 remained indeterminate, one became RIBA negative, and one gained (subject 11) a further band on the RIBA test (see table 3.1).

3.3. Ex-vivo IFN-  $\gamma$  ELISpot responses

Figure 3.1 shows the results of ELISpot analysis of T cells reactive to HCV in indeterminate donors and in healthy, unscreened controls tested with HCV core peptide pools 1-4 and NS3-5 proteins. Viral-specific ELISpot responses were obtained in 15 of 30 RIBA-indeterminate donors and none in 14 controls ( $p=0.0013$ ; Fisher's exact test). A response to at least one of the tested antigens was regarded as positive (table 3.2).



**Figure 3.1. Proportion of RIBA-indeterminate individuals and controls showing T-cell reactivity specific to hepatitis C virus by interferon- $\gamma$  ELISpot.**

Error bar= 95% CI, calculated by binomial exact method (Stata version 7).

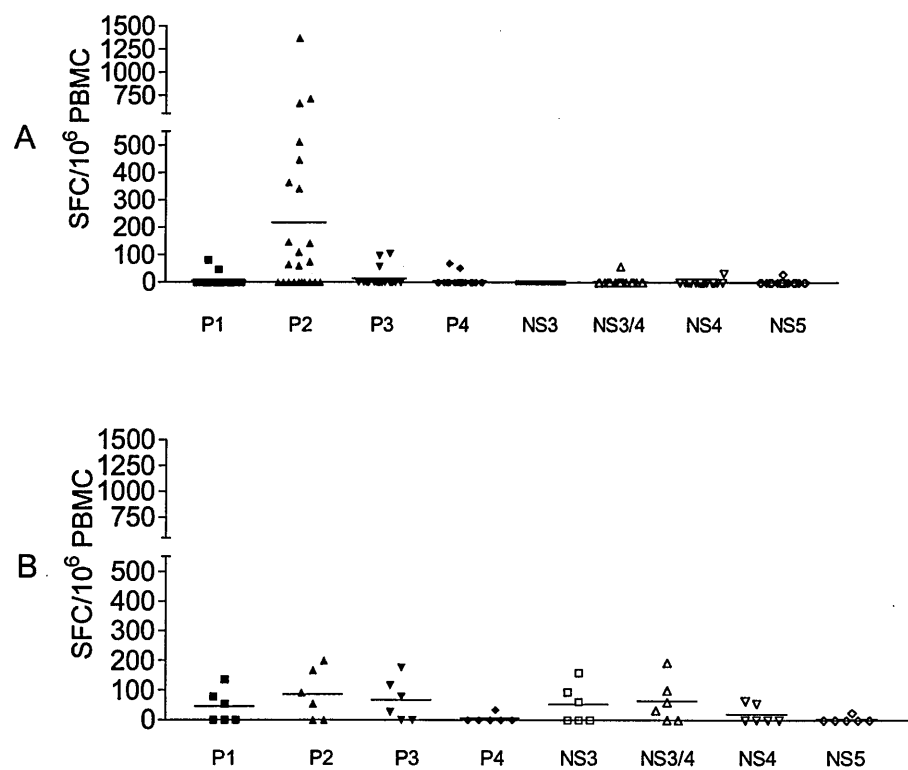
### ***3.3.1. Responses in RIBA-indeterminate donors are mainly focused on core peptides***

Next, I analysed the fine specificity of these responses. In the indeterminate group, 13 of 15 responders reacted mainly to core peptides, with minimum responses to NS3–NS5 proteins. The most commonly recognised pool of core peptides was pool 2, a region spanning 60 amino acids (51–110; figure 3.2A). Fig. 3.2C shows an example of such a response. Interestingly, these target responses differ from the antibody responses in the RIBA assays. Here, the main target of the antibodies was the NS3 (c33).

### ***3.3.2. Responses in resolved individuals are broader than in RIBA-indeterminate group***

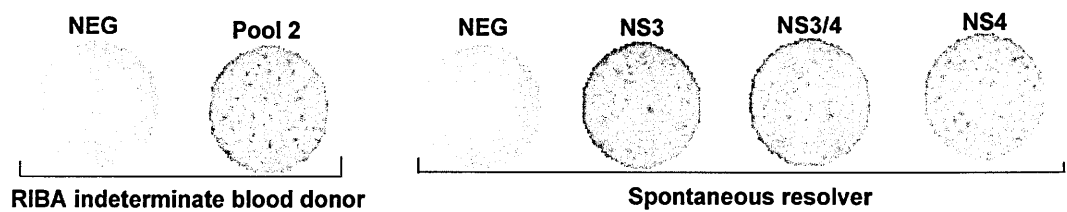
I compared these responses with those of six spontaneous resolvers, who gave positive RIBA results but did not have detectable concentrations of viral RNA. As expected from other studies, this group had strong reactivity (in five resolvers) to a range of tested antigens (median four antigens, range zero–six antigens; figure 3.2B) (Barnes et al., 2002; Lauer and Walker, 2001; Takaki et al., 2000). Overall, the specificity of responses differed between indeterminate and spontaneous resolver groups. Responses in the resolver group were broader, commonly targeting non-structural proteins (two of 30 [indeterminates] vs five of six responses [resolvers],  $p=0.0003$ ; Fisher's exact test). Fig. 3.2.C shows an example of such a response.





**Figure 3.2 A+B. Specific T cell response to tested antigens in ELISpot.**

A, responses from indeterminate blood donors (n=30). B, responses from spontaneous resolvers (n=6). SFC/10<sup>6</sup> PBMC= spot forming cells per million peripheral blood mononuclear cells. P1, P2, P3, P4 represent pools of viral core peptides of HCV. NS3, NS3/4, NS4, and NS5 represent recombinant and non-structural proteins of the virus.



**Figure 3.2C. IFN-γ ELISpot examples.**

Shown for one RIBA-indeterminate blood donor (subject 21) with a core pool 2 response and two spontaneous resolvers with responses to NS3 (subject R2), NS3/4 and NS4 (subject R3), respectively.

Interestingly, the magnitude of responses to the core pool 2 was higher in the RIBA-indeterminate group when compared with the spontaneously resolved group. However, this was not significant (Mann-Whitney test).

#### 4. Discussion

These results indicate that a substantial proportion of indeterminate donors have memory T cell populations that can react with HCV antigens. Their detected responses are focused on the core region of the virus. These focused responses could be remnants of a broadly directed response. Because this response declines in magnitude over time, only a partial response can be detected. Alternatively, they might represent a particular variant of the protective response that eliminates the virus. Such responses could arise in situations with limited antigen exposure or potentially when the virus replicates poorly.

Although this study was cross-sectional, I have seen another individual in whom a RIBA-indeterminate state had been recorded long term. This individual seroconverted (as indicated by EIA testing) 6 years before, during an acute hepatitic illness, but RIBA test results at this time and 3 years later were indeterminate. PCR results were consistently negative and ELISpot testing revealed a robust response to core antigens of the virus (pool 2, 110 spots per million) with a weaker *ex vivo* response to NS3 (40 spots per million, confirmed by extensive studies of cultured cells) (Harcourt, 2004).

Thus an indeterminate RIBA and positive ELISpot state might be seen for a long time after documented acute viral infection of HCV. T cell responses in RIBA-indeterminates could represent cross-reactivity with a non-viral antigen, as previously reported (Wedemeyer et al., 2001). However, the fact that these responses are not seen in controls but are seen in confirmed infected patients suggests that responses are probably linked to exposure to HCV. The responses also target a range of different peptides within the core region (data not shown) as well as non-core regions, so any cross-reactivity would need to be quite extensive. T cell responses were not detectable in all individuals studied (15/30 RIBA-indeterminate individuals were negative). The ELISpot analyses might have been limited by the use of genotype 1 prototype peptides and proteins of the virus, which might not have detected responses in those with non-genotype 1 infection. Some of these 15 donors with no T cell responses to HCV might have been infected with another genotype and therefore could have been missed here. It would have been interesting to see whether the use of antigens from HCV sequences other than the genotype 1 could have increased the response frequency in this group. Unfortunately, we did not have any antigens of other HCV genotypes available to test this hypothesis. Alternatively, RIBA indeterminate serology in these 15 individuals with no detectable T cell responses could have been “false positive”. Additionally, even though the ELISpot can identify about one cytokine secreting cell per 100,000 peripheral-blood mononuclear cells, more sensitive tests might have revealed additional specificities in some individuals. For example, the use of class II tetrameric complexes showed a response directed at NS3 in an individual from the spontaneous resolver group whose ex-vivo ELISpot was negative (Day et al., 2003).

Long-term culture of T cells with specific antigens might also amplify populations that are undetectable in ex-vivo ELISpot tests. Finally, the use of overlapping peptides for the entire genome of HCV, rather than just the core, might enhance the detection of ex-vivo responses against NS proteins. Here, the reason why the responses in core peptides were stronger than for NS proteins might be due to the preparation of these different antigens. The core peptides stimulated in the ELISpot for 18 hours are more likely to induce T cell responses within this period of time as they do not have to undergo the conventional MHC peptide processing pathway. They already can bind to the peptide binding groove of either the MHC class I or class II APCs. However, in the case of the NS proteins, these have to go through the MHC antigen processing pathway, with cleavage of the proteins into peptides before T cell responses can be induced. Therefore, with only 18 hours of stimulation in the ELISpot responses could be underestimated. But this can be put only as a speculation, as in the spontaneous resolver group responses were not only observed in the core peptides but also to a similar extent in the NS proteins.

Unfortunately, due to the lack of cryopreserved PBMC we were not able to repeat some of these assays in specific individuals. Also, it would have been interesting to see whether responses to the core peptide pool 2 could have been further restricted to any one peptide by extending the assays. The lack of PBMC was also the reason for not being able to perform T cell subset depletions to see whether the responses observed were mainly CD4<sup>+</sup> or CD8<sup>+</sup> T cell restricted. However, CD8 depletion ELISpot assays in spontaneously resolved individuals confirmed these were mainly CD4<sup>+</sup> derived responses.

Examples of depletion assays are further described and shown in chapter 4.

Interestingly, T cell responses in the ELISpot were different from the antibody responses in the RIBA assays. Here, the main target of the antibodies was the NS3 (c33). One explanation could be that initially antibody responses were broader, thus targeting also the Core region, but antibody reactivity declined over time after spontaneous resolution of infection.

Although the T cell ELISpot is not presently used as a clinical test, such analyses in the future could shed further light on the status of such indeterminate donors as well as the role of T cell control in hepatitis C (as well as other infections). These data suggest that RIBA-indeterminate donors should be included in future studies of HCV natural history, because the true rate of spontaneous viral clearance could be underestimated. This means that the spontaneous HCV clearance rate may not be as low as only 20% as has been previously estimated, but may be even up to 50%. Finally, these findings suggest that indeterminate blood donors should be counselled appropriately, with respect to potential previous HCV exposure. From a clinical point of view, as they have cleared the virus, one should give them the reassurance that they are not infectious to anyone else (e.g. to their partners).

## 5. Further work

This study was based on work from a pre-defined sample of RIBA-indeterminate cases. Although these are most commonly identified among blood donor screening, occasionally RIBA-indeterminate individuals are found on routine screening in risk groups. Local records were therefore analysed to see if the group could be enlarged and more detailed investigation performed. In particular I wished to see if these responses could be mapped down to single peptides.

Of two additionally tested patients, one had ELISpot responses not only to the core peptide pool 2 but also to core peptide pool 4 (Pat. Ch.D: Local 1). When single peptides were broken down, the stimulating peptide in this patient was mapped to peptide 18 (aa 171-190:GCSFSIFLLALLSCLTPAS). The second patient showed responses to the core peptide pool 3 (Pat. A.F: Local 2). Due to the lack of cells, further mapping of peptides in this patient was not possible.

These data are not conclusive but show that

- a) similar findings may be obtained in a separate patient group and
- b) responses may be mapped down to single peptides.

## 6. Conclusions

1. RIBA-indeterminate donors display evidence of cellular immunity to HCV and therefore prior exposure to antigen.
2. The proportion of individuals who spontaneously clear HCV may be higher than previously estimated.
3. Appropriate counselling with respect to previous HCV exposure is necessary.

## CHAPTER 4

### PREFERENTIALLY LOWER IL-2 SECRETING CD4<sup>+</sup> T HELPER CELLS IN CHRONIC HCV INFECTION

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## 1. Abstract

**Background and aims:** Hepatitis C virus (HCV) becomes persistent in the majority of infected individuals. In doing so, it evades host adaptive immune responses, although the mechanisms responsible for this evasion are not clear. Several groups have demonstrated weak or absent HCV-specific CD4<sup>+</sup> T cell responses during chronic HCV infection using proliferation assays and, more recently Class II tetramers. However, the functional status of HCV-specific CD4<sup>+</sup> T cells in resolved and persistent infection is poorly understood.

**Methods and Results:** Using interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin 2 (IL-2) enzyme-linked immunospot assays (ELISpot), cytokine secretion patterns were analysed in chronically infected patients and compared with those from patients with resolved infection. In the spontaneous resolver group, strong IL-2 secretion in relation to IFN- $\gamma$  secretion was observed. However, in the persistently infected group, there were significant fewer IL-2 secreting cells when compared to IFN- $\gamma$  secreting cells. *In vitro* addition of IL-2 had a substantial effect in restoring CD4<sup>+</sup> T cell activity.

**Conclusions:** In conclusion, failure of IL-2 secretion, as opposed to physical deletion or complete functional unresponsiveness, appears to be an important determinant of the status of CD4<sup>+</sup> T cell populations in chronic HCV infection. Reduced IL-2 secretory capacity may lead to disruption of IFN- $\gamma$  production and proliferative function *in vivo* – a status that characterises the cellular immune response in both CD4<sup>+</sup> and CD8<sup>+</sup> T cell compartments in chronic disease.

## 2. Introduction

Hepatitis C virus (HCV) is a major cause of morbidity and mortality worldwide. Persistent infection is readily established and is associated with the evolution of liver fibrosis, cirrhosis, and hepatocellular carcinoma (Lauer and Walker, 2001; McHutchison, 2004). The mechanisms behind viral persistence are poorly understood (Cerny and Chisari, 1999; Rehmann, 2000). HCV is thought to evade host innate immune responses through, for example, interactions with interferon signalling molecules (Francois et al., 2000; Gale et al., 1998; Taylor et al., 1999). Adaptive immune responses do, however, play a significant role in viral control (Bukh et al., 2001; Chang et al., 2001a; Diepolder et al., 1995b; Gerlach et al., 1999; Naoumov, 1999; Ward et al., 2002). A fraction of individuals (about 20%) appears to be able to contain the virus after initial exposure and develop a stable control of viral replication state. In these individuals, there is functional and genetic evidence that CD8<sup>+</sup> and CD4<sup>+</sup> T cells play a role in prevention of persistence (Diepolder et al., 1995b; Gruener et al., 2000; Lechner et al., 2000).

Once persistent infection is established, T cell responses in blood appear to be weaker than in patients who spontaneously resolve infection (Day et al., 2002). In particular, loss of CD4<sup>+</sup> T cell proliferative responses appears to be a hallmark of persistent infection (Chang et al., 2001a; Ulsenheimer et al., 2003; Wedemeyer et al., 2002).

Failure to establish proliferative T cell responses during acute infection may contribute to the establishment of persistence (Gerlach et al., 1999), although, in cross-sectional studies, it is not clear whether such changes are cause or effect.

Failure to detect HCV-specific T cells in proliferation assays may result from deletion of such cells, functional anergy, or evolution of effector cell populations that lack proliferative capacity (Wedemeyer et al., 2002). In HIV infection, recent data suggest that, in the presence of high viral loads, HIV-specific CD4<sup>+</sup> T cell populations do exist but lack proliferative capacity (Younes et al., 2003); this status is associated with the production of IFN- $\gamma$  upon antigen stimulation, but little or no IL-2. Once antigen loads are lowered through drug treatment, cell proliferation and IL-2 secretion can both be restored.

In HCV infection, even in patients with chronic disease for decades, treatment with anti-viral regimes such as interferon- $\alpha$ /ribavirin combination therapy can lead to restoration of CD4<sup>+</sup> T cell proliferative capacity, and to a lesser extent, IFN- $\gamma$  secretion (Barnes et al., 2002; Cramp et al., 2000; Tam et al., 1999). Therefore, IFN- $\gamma$  and IL-2 secretion by HCV-specific CD4<sup>+</sup> T cell responses upon antigen stimulation were compared in individuals who had either chronic or spontaneously controlled HCV.

### 3. Results

#### 3.1. Study subjects

For the analysis of HCV-specific T cell responses, blood was obtained from 23 untreated patients (Table 1; CH1- CH23) with persistent HCV infection (RNA+), defined as the detection of HCV RNA by RT-PCR on at least two consecutive occasions 6 months apart (detection limit of 300 HCV RNA copies/ml of plasma; v2.0 Amplicor assay, Roche Diagnostics Ltd, Basel, Switzerland). Blood was also obtained from 11 patients (Table 4.1; R1- R11) with spontaneously resolved HCV infection (RNA-), defined as the absence of detectable HCV RNA by RT-PCR in the presence of HCV antibodies (Abbott HCV EIA 3.0, Abbott Laboratories, Abbott Park, Illinois) on at least two consecutive occasions 6 months apart. Thirteen healthy individuals from my lab were also recruited as negative controls (7 females, 6 males; age range 23-53) and tested for IFN- $\gamma$  ELISpot. In 11 of them IL-2 ELISpots were also performed. Biopsies on RNA+ patients were scored using the method of Ishak to assess fibrosis (range 0-6) and inflammation (range 0-18) (Ishak et al., 1995).

For detailed methods see methods chapter 2.

Subject	Age	Sex	Staging (0-6)*	Grading (0-18)*	ALT (normal <45 U/L)	Genotype
CH1	29	M	2	4	104	3a
CH2	43	M	-	-	25	1a
CH3	58	F	1	3	27	2b
CH4	37	M	1	1	78	3a
CH5	40	F	-	-	11	1a
CH6	49	F	3	2	47	1a
CH7	35	M	3	9	119	1a
CH8	41	F	1	4	26	1
CH9	42	M	-	-	499	3a
CH10	55	M	-	-	55	1a
CH11	37	F	1	4	40	1b
CH12	65	M	3	5	445	1b
CH13	24	F	1	4	31	1
CH14	40	M	1	3	194	1a
CH15	44	M	2	6	267	4c
CH16	50	M	0	1	47	4
CH17	43	F	1	3	28	1
CH18	46	M	1	3	66	3a
CH19	22	M	-	-	78	3a
CH20	41	M	-	-	68	3a
CH21 ★ (CH4)	50	M	6	n.a.	182	3a
CH22	75	F	2	4	32	1
CH23	48	M	2	1	30	1a
R1 ★ (R8)	46	F				
R2	32	M				
R3 ★ (R5)	53	M				
R4	56	F				
R5 ★ (R3)	75	F				
R6 ★ (R4)	44	F				
R7 ★ (R2)	43	M				
R8 ★ (R7)	48	M				
R9 ★ (R6)	41	F				
R10★(R12)	49	F				
R11	60	M				

Table 4.1. Baseline characteristics of the patients

\*Staging (fibrosis) and grading (inflammation) was clinically scored according to Ishak’s Scoring method (Ishak); n.a., result not available; -, no biopsy performed; ALT values in resolved individuals were tested normal.

★, these patients have been used and retested again in chapter 5 where different ID numbers have been allocated to them (IDs in brackets).

ID	Cytokine	TT	CMV	Pool1	Pool2	Pool3	Pool4	NS3	NS3/4	NS4	NS5	P1-4	NS3-5	P1-4 + NS3-5
CH1	IFN-g	47,5	0	0	47,5	305	0	0	0	0	0	352,5	0	352,5
CH1	IL-2	52,5	0	0	0	67,5	0	0	0	0	0	67,5	0	67,5
CH2	IFN-g	320	0	0	0	27,5	0	0	0	0	0	27,5	0	27,5
CH2	IL-2	0	0	0	0	0	0	0	0	0	0	0	0	0
CH3	IFN-g	0	0	0	37,5	25	0	0	37,5	0	0	62,5	37,5	100
CH3	IL-2	22,5	0	0	0	0	0	0	0	0	0	0	0	0
CH4	IFN-g	0	630	0	0	0	0	0	0	0	0	0	0	0
CH4	IL-2	0	100	0	0	0	0	0	0	0	0	0	0	0
CH5	IFN-g	225	370	0	0	20	0	0	0	0	0	20	0	20
CH5	IL-2	15	45	0	0	0	0	0	0	0	0	0	0	0
CH6	IFN-g	235	0	0	0	0	0	0	0	0	0	0	0	0
CH6	IL-2	425	0	0	0	0	0	0	0	0	0	0	0	0
CH7	IFN-g	40	1000	0	0	0	0	0	0	0	0	0	0	0
CH7	IL-2	0	175	0	0	0	0	0	0	0	0	0	0	0
CH8	IFN-g	47,5	0	0	0	0	0	0	0	0	0	0	0	0
CH8	IL-2	27,5	0	0	0	0	0	0	0	0	0	0	0	0
CH9	IFN-g	0	25	75	122,5	175	37,5	27,5	0	0	0	409,5	28	437,5
CH9	IL-2	0	0	0	0	0	0	0	0	0	0	0	0	0
CH10	IFN-g	45	1000	47,5	130	127,5	65	35	25	40	0	370	100	470
CH10	IL-2	0	132,5	27,5	45	27,5	0	0	0	0	0	100	0	100
CH11	IFN-g	195	0	0	0	0	0	0	0	0	0	0	0	0
CH11	IL-2	245	0	0	0	0	0	0	0	0	0	0	0	0
CH12	IFN-g	0	117,5	0	0	27,5	0	0	0	0	0	27,5	0	27,5
CH12	IL-2	17,5	0	0	0	0	0	0	0	0	0	0	0	0
CH13	IFN-g	0	1000	0	0	0	0	0	0	0	0	0	0	0
CH13	IL-2	77,5	130	0	0	0	0	0	0	0	0	0	0	0
CH14	IFN-g	202,5	0	0	0	0	0	0	0	0	0	0	0	0
CH14	IL-2	0	0	0	0	0	0	0	0	0	0	0	0	0
CH15	IFN-g	0	1000	0	0	75	0	0	42,5	0	0	75	42,5	117,5
CH15	IL-2	0	312,5	0	0	20	0	0	17,5	0	0	20	17,5	37,5
CH16	IFN-g			0	0	0	0					0		0
CH16	IL-2			0	0	0	0							0
CH17	IFN-g	120	443	0	20	40	0	0	0	0	0	60	0	60
CH17	IL-2	55	135	0	0	0	0	0	0	0	0	0	0	0
CH18	IFN-g			155	0	85	0		0			240	0	240
CH18	IL-2			0	0	0	0		0			0	0	0
CH19	IFN-g			0	0	0	0		0			0	0	0
CH19	IL-2			0	0	0	0		0			0	0	0
CH20	IFN-g			388	0	0	63		0			451	0	451
CH20	IL-2			0	0	0	0		0			0	0	0
CH21	IFN-g			0	55	75	0		0			130	0	130
CH21	IL-2			0	30	0	0		0			30	0	30
CH22	IFN-g			0	0	25	0	0	0	0	0	25	0	25
CH22	IL-2			0	0	0	0	0	0	0	0	0	0	0
CH23	IFN-g			0	0	0	0	0	0	0	0	0	0	0
CH23	IL-2			0	0	0	0	0	0	0	0	0	0	0
R1	IFN-g	127,5	1000	52,5	0	32,5	37,5	65	0	65	35	187,5	100	287,5
R1	IL-2	47,5	697,5	0	0	0	0	0	0	40	0	0	40	40
R2	IFN-g	27,5		0	0	15	32,5	0	0	0	0	47,5	0	47,5
R2	IL-2	0		0	0	0	0	0	0	0	0	0	0	0
R3	IFN-g	0	150	0	0	0	0	0	235	232,5	0	0	467,5	467,5
R3	IL-2	0	0	0	0	0	0	0	50	57,5	0	0	107,5	107,5
R4	IFN-g	57,5	1000	45	17,5	20	25	30	20	25	17,5	107,5	92,5	200
R4	IL-2	27,5	0	0	0	0	0	0	0	0	0	0	0	0
R5	IFN-g	75	1000	30	65	80	0	62,5	37,5	0	0	175	100	275
R5	IL-2	195	332,5	145	125	207,5	0	75	40	0	0	477,5	115	592,5
R6	IFN-g	27,5	0	0	182,5	55	25	0	0	0	92,5	262	93	355
R6	IL-2	0	0	0	57,5	35	0	0	0	0	0	92,5	0	92,5
R7	IFN-g		0	30	17,5	20	0	0	0	0	0	67,5	0	67,5
R7	IL-2		0	0	0	0	0	0	0	0	0	0	0	0
R8	IFN-g	0	0	0	0	0	0	0	50	35	0	0	85	85
R8	IL-2	0	0	0	0	0	0	0	25	42,5	0	0	67,5	67,5
R9	IFN-g	67,5	332,5	0	0	0	0	0	100	35	55	0	190	190
R9	IL-2	25	135	20	0	0	0	22,5	50	50	40	20	162,5	182,5
R10	IFN-g	227,5	735	80	170	140	160	0	0	0	0	550	0	550
R10	IL-2	50	0	91,5	110	110	115	0	0	0	0	426,5	0	426,5
R11	IFN-g	0	350	22,5	0	0	0	0	0	0	0	22,5	0	22,5
R11	IL-2	22,5	47,5	0	0	0	0	0	0	0	0	0	0	0

Table 4.2. ELISpot raw data in chronic and resolved individuals.

Data are shown for each individual tested for HCV core peptides and NS3-5 proteins.

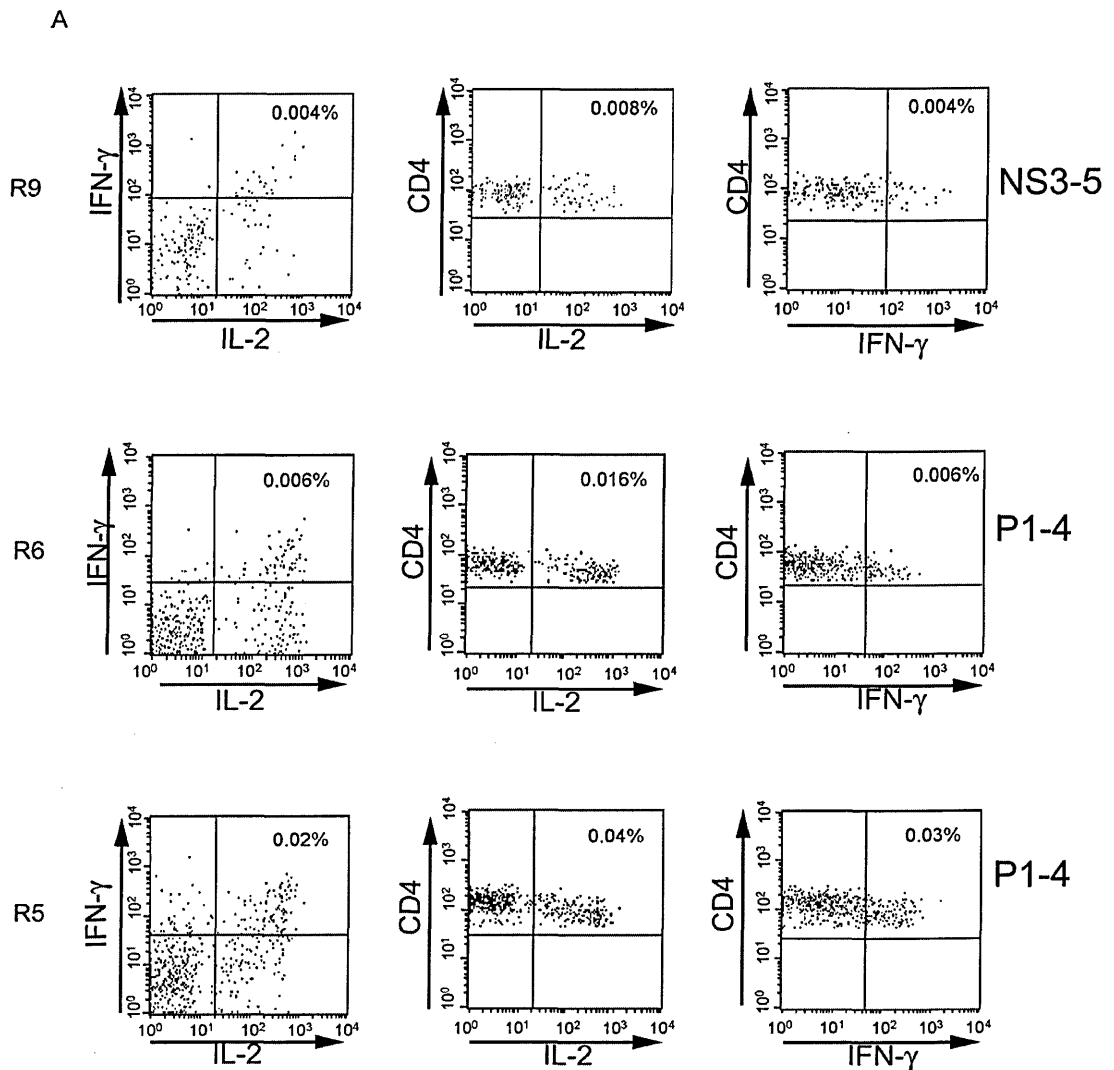
HCV-specific responses in healthy controls were negative. TT and CMV-L were also tested as irrelevant test antigens in the majority of the patients. Responses are shown as SFC/10<sup>6</sup> PBMC.

### **3.2. Ex vivo cytokine release in spontaneously resolved (RNA-) and chronically infected individuals (RNA+)**

#### **3.2.1. HCV-specific CD4<sup>+</sup> T cell responses in IFN- $\gamma$ and IL-2 secretion assays**

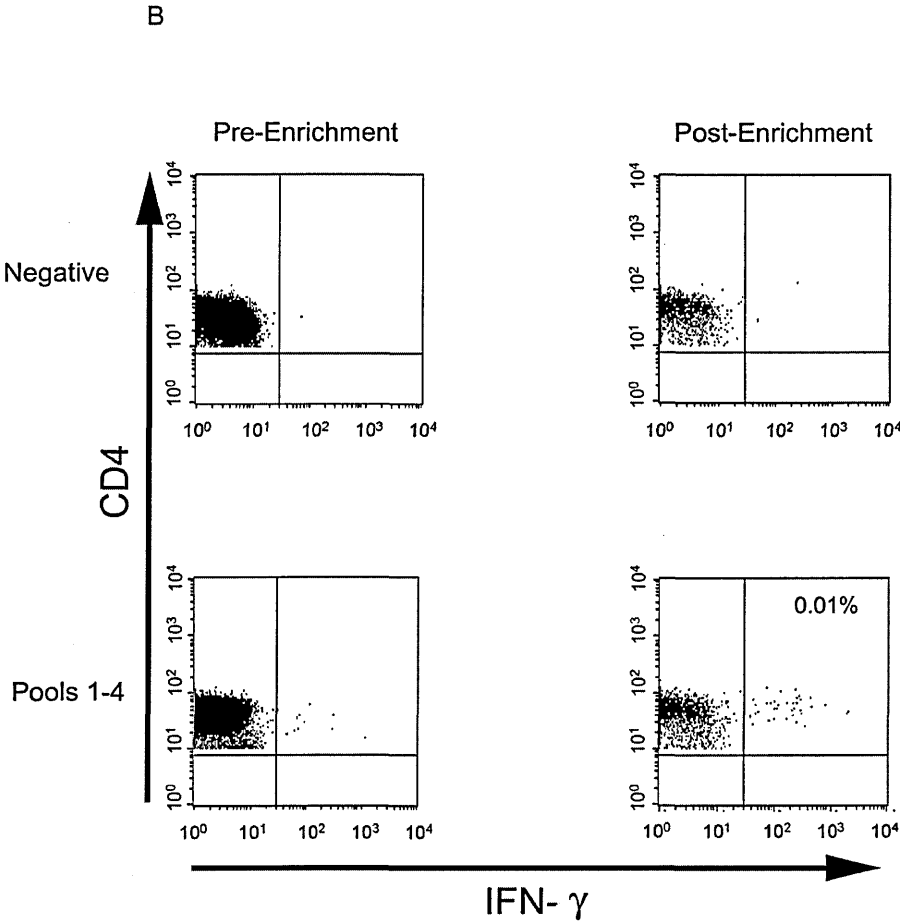
PBMC isolated from individuals with spontaneously resolved HCV infection (RNA-) were stimulated with HCV antigens and their ability to secrete IFN- $\gamma$  and IL-2 was analysed using a cytokine secretion assay. Strong CD4<sup>+</sup> T cell responses were detected in 8/8 individuals tested (Fig. 4.1A). Due to lack of cells the three other resolved individuals were not tested in this assay. An example of CD4<sup>+</sup> T cells secreting cytokine in response to HCV peptides visible on flow cytometry after magnetic bead enrichment from a RNA+ subject is shown in Fig. 4.1B.





**Figure 4.1A. Analysis of cytokine secretion by antigen-specific CD4<sup>+</sup> T cells.**

Analysis of function of HCV-specific CD4<sup>+</sup> T cells shown from three representative patients with spontaneously resolved HCV infection (R5, R6, R9). PBMC were stimulated either for 16 hours with a pool of recombinant NS3, NS4 and NS5 (NS3-5) antigens (R9) or for 6 hours with a pool of the core peptide pools 1-4 (R5+ R6)), respectively, and isolated with an IL-2 and/or IFN- $\gamma$  cell enrichment and detection assay. The percentage in the upper right quadrants of each FACS plot represents cytokine-secreting CD4<sup>+</sup> T cells among enriched cells calculated from input cell number (see methods); left hand FACS plots: combined IL-2/IFN- $\gamma$  double positive CD4<sup>+</sup> T cells; middle FACS plots: IL-2 producing CD4<sup>+</sup> T cells; right hand FACS plots: IFN- $\gamma$  producing CD4<sup>+</sup> T cells. An example of a negative control is shown in Fig. 4.1.B.

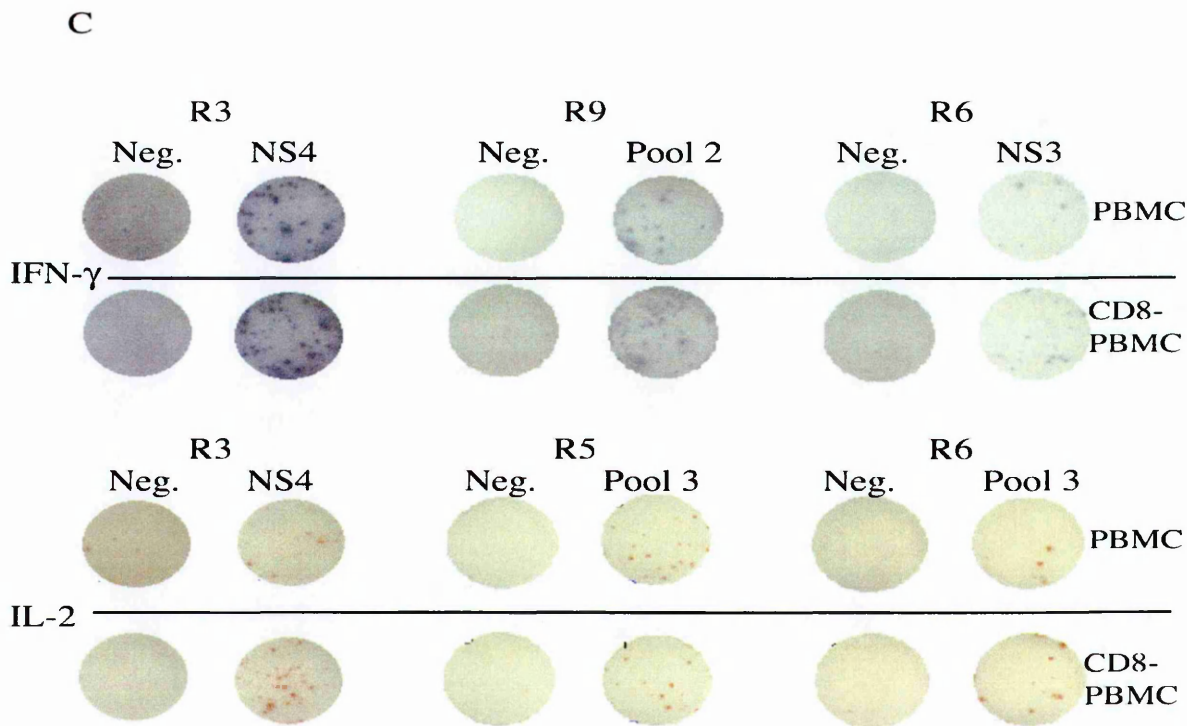


**Fig. 4.1.B Analysis of cytokine secretion by antigen-specific CD4<sup>+</sup> T cells.**

PBMC of a chronic HCV patient were stimulated for 6 hours with core peptide pools 1-4. The responding cells were stained and isolated according to secretion of IFN- $\gamma$  using the IFN- $\gamma$  secretion assay-cell enrichment and detection kit. Upper plots: Negative control, lower plots: Frequency after stimulation with core peptide pools 1-4.

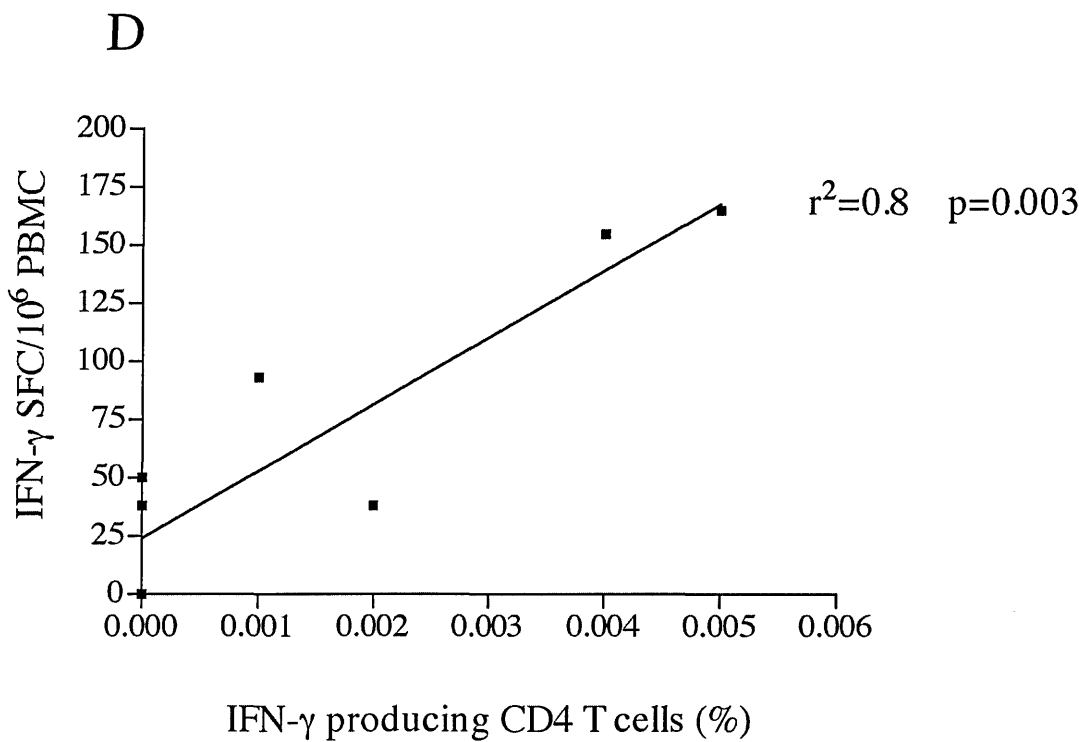
### 3.3. HCV-specific CD4<sup>+</sup> T cell responses in IFN- $\gamma$ and IL-2 *ELISpot* assays

In order to screen larger patient numbers and multiple antigens simultaneously and avoid the requirement for positive selection using magnetic beads, IL-2 and IFN- $\gamma$  ELISpot assays were used. The magnitude of the T cell response against HCV in patients CH1-23 and R1-11 was measured using an ELISpot assay, as previously described (Barnes et al., 2002) and discussed in Patients and Methods, to assess responses to Core, NS3, NS3/4, NS4 and NS5. Depletion of CD8<sup>+</sup> cells from PBMC confirmed that in all the samples tested (in 6 resolved individuals), responses to the HCV antigens for both IFN- $\gamma$  and IL-2 were independent of CD8<sup>+</sup> T cells (Fig. 4.1C). Similarly, a correlation between CD4<sup>+</sup> T cells secreting cytokine (as measured by the flow cytometric assay) and the ELISpot was observed (Fig. 4.1D+E, linear regression). These assays together suggest the majority of the responses observed were due to CD4<sup>+</sup> T cells.



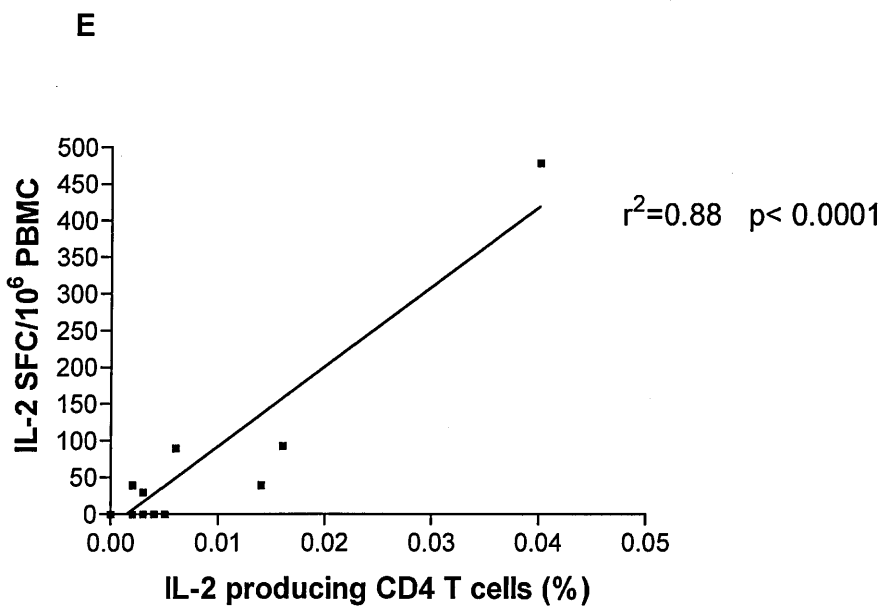
**Fig. 4.1.C. CD4<sup>+</sup> T cell mediated IFN-γ and IL-2 responses in ELISpot assays.**

Depletions were performed in 6 resolved individuals. Examples of four individuals are shown. Upper two panels show an IFN-γ ELISpot against NS4 (R3), core peptide pool 2 (R9) and NS3 (R6) in three representative HCV resolved patients before and after CD8 depletion, whereas the lower two panels show an IL-2 ELISpot against NS4 (R3) and core peptide pool 3 (R5+R6) before and after CD8 depletion (purple spots= IFN-γ, red spots= IL-2). Compared to table 4.2., for this aim, ELISpot assays in these individuals were repeatedly set up, some of which were from cryostored PBMC. Therefore, responses shown here might differ from those shown in table 4.2. Example for correcting for the number of CD4 T cells after CD8 depletion: Number of IL-2 SFC/10<sup>6</sup> PBMC in patient R3 tested in NS4= 45 SFC/200,000 in CD8 depleted PBMC (corrected = 113 SFC/10<sup>6</sup> PBMC, compared to 55/10<sup>6</sup> in ex vivo sample). The formula for correcting for the number of CD4 T cells after CD8 depletion is described in material and methods.



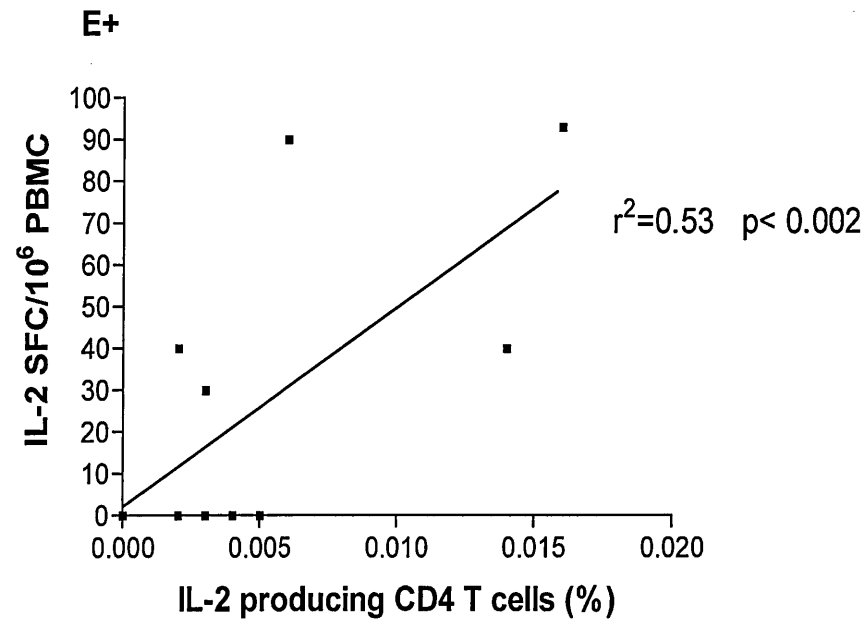
**Fig. 4.1.D. IFN- $\gamma$  ELISpot and IFN- $\gamma$  cytokine secretion assay correlation.**

Shown for 8 resolved individuals tested against the non-structural proteins NS3-5. In this assay, both responses are tested after 18 hours of exposure to antigen. Although ELISpot assays were performed in 11 resolved individuals, only 8 could be compared with cytokine secretion assays that were performed only in 8 individuals. Only seven dots are visible on the graph as 2 dots go through zero. Similar relations were also seen for analysis of responses against all epitopes (core P1-4+NS3-5) for IFN- $\gamma$  ( $r^2=0.3$ ,  $p=0.038$ ).



**Fig. 4.1.E. IL-2 ELISpot and IL-2 cytokine secretion assay correlation.**

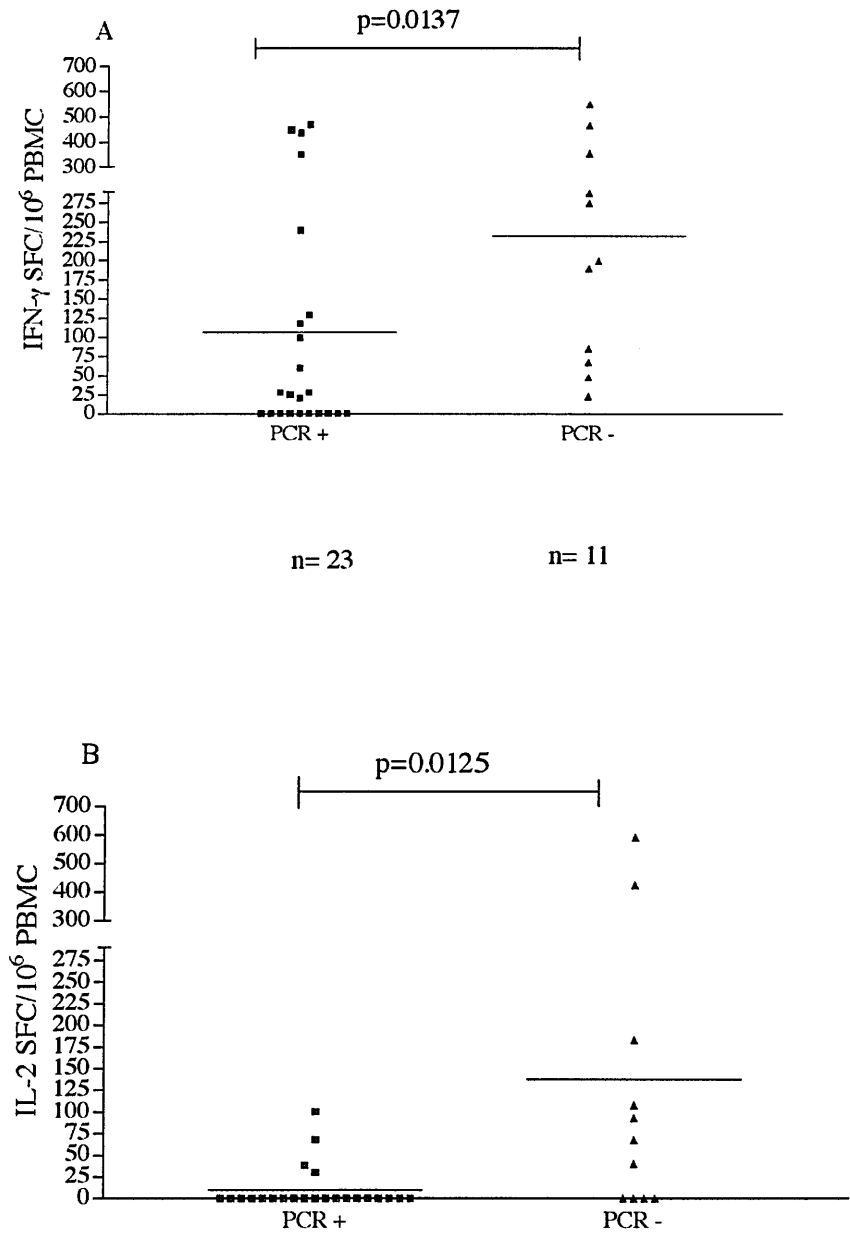
Shown for 8 resolved individuals tested against core peptides pools 1-4 and the non-structural proteins NS3-5, displayed side by side (16 spots in total). Only 11 spots are visible as 5 other spots go through zero. The relation is still significant even if the individual with values 478 SFC/10<sup>6</sup> PBMC and 0.04% is removed ( $r^2=0.53$ ,  $p=0.002$ ), as shown in the figure below:



**3.3.1. Larger cytokine responses in RNA- group compared to RNA+ group**

Having validated the cytokine-secretion assays, I compared HCV-specific CD4<sup>+</sup> T cell responses between RNA+ and RNA- individuals. Overall, significantly larger responses could be measured using the IFN- $\gamma$  ELISpot in the RNA- group than in the RNA+ group. Interestingly, the response difference across all antigens, although significant, was only about 2.2 fold greater (mean 232 spots per million vs. mean 107 spots per million,  $p=0.0137$ , Mann-Whitney test; Fig. 4.2A). A positive IFN- $\gamma$  response was detected in all (11/11) RNA- individuals, but only in 13/23 RNA+ individuals ( $p=0.0135$ , Fisher's exact test). These data are consistent with previous analyses indicating weak CD4<sup>+</sup> T cell responses in RNA+ individuals using proliferative assays, but the magnitude of the differences is smaller than might be predicted from such analyses.

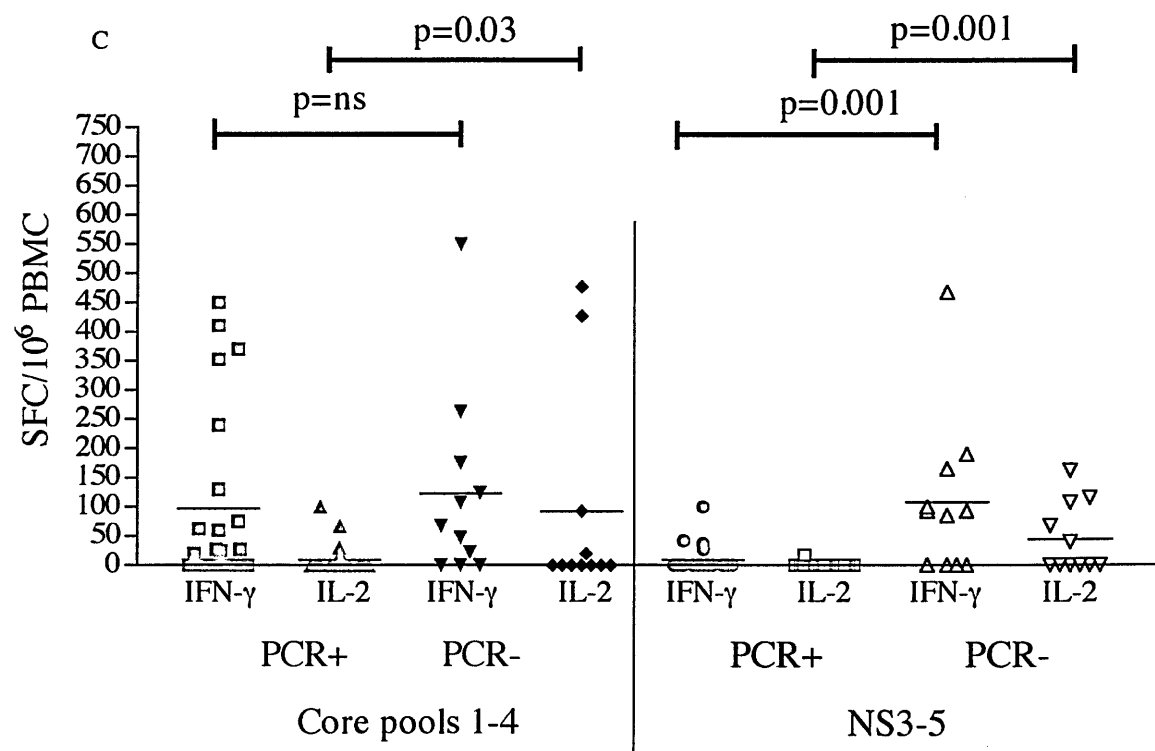
IL-2 ELISpots were also performed in parallel. In the RNA- group, responses across all antigens were similar in overall magnitude to IFN- $\gamma$ ; however, in the RNA+ group, only weak responses were obtained (mean 138 spots per million vs. mean 10 spots per million;  $p=0.0125$ , Mann-Whitney test; Fig. 4.2B). The majority of individuals within the RNA+ group had no detectable IL-2 ELISpot response at all (4/23 RNA+ vs. 7/11 RNA-;  $p=0.016$ , Fisher's exact test). No responses using either IFN- $\gamma$  or IL-2 ELISpots were detectable in the healthy negative control group (data not shown).



**Figure 4.2. A+B. Magnitude of HCV-specific T cell responses as determined by ELISpot for both IFN- $\gamma$  and IL-2.** PBMC were stimulated using HCV core peptide pools 1-4 and HCV non-structural proteins NS3, NS3/4, NS4 and NS5 as described in the methods. Overall responses of IFN- $\gamma$  and IL-2 producing PBMC comparing 23 chronic HCV patients (RNA+) with 11 HCV resolvers (RNA-). Responses represent the mean of duplicate wells after subtraction of the background and have been combined for core peptides and NS proteins (the sum of the two)(see also Table 4.2, column P1-4+NS3-5). Mann-Whitney test was used. PCR+ = RNA+; PCR- = RNA- .



The differences between the groups were maintained when responses of different specificities were analysed (Fig. 4.2C). RNA- individuals maintained broad responses, which were detectable against all antigens tested for both IFN- $\gamma$  and IL-2 (*P* value not significant). IFN- $\gamma$  responses against core peptides were common in the RNA+ group, but were significantly weaker using the IL-2 assay ( $p=0.006$ , paired t-test). The IFN- $\gamma$  responses in the RNA+ group against NS3-5 were less common (4/23) and an IL-2 response was detectable in only one individual.



**Fig. 4.2. C.** Overall responses in IFN- $\gamma$  and IL-2 ELISpots broken down by antigen. The responses to core peptides and NS proteins are displayed separately. The shown *p* values were achieved using the Mann-Whitney test. PCR+ = RNA+; PCR- = RNA- .

3.3.2. Relation between genotype and HCV-specific T cell responses

As HCV-specific IFN- $\gamma$  T cell responses were detectable in 13/23 RNA+ individuals, we were interested to see whether a relation between HCV genotype and T cell responses existed in these patients. Of these 23 patients, 13 were infected with HCV genotype 1, 1 with genotype 2, 7 with genotype 3 and 2 with genotype 4 (Table 4.1). IFN- $\gamma$  T cell responses  $>225$  SFC/ $10^6$  PBMC were seen in 5/23 individuals (Fig. 4.2.A+D); interestingly, 4 of these had genotype 3. Responses were significantly higher in individuals infected with genotype 3 when compared to those of patients infected with genotype non-3 (Fig. 4.2.D;  $p=0.0124$ ; non-paired t test). It would have been interesting to see whether there was a relation between T cell responses and viral load. Unfortunately, viral loads were not available/tested at the timepoint when PBMC were obtained from these patients.

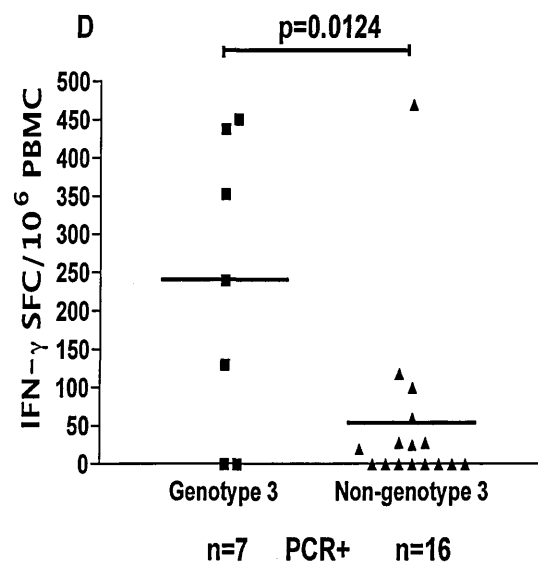
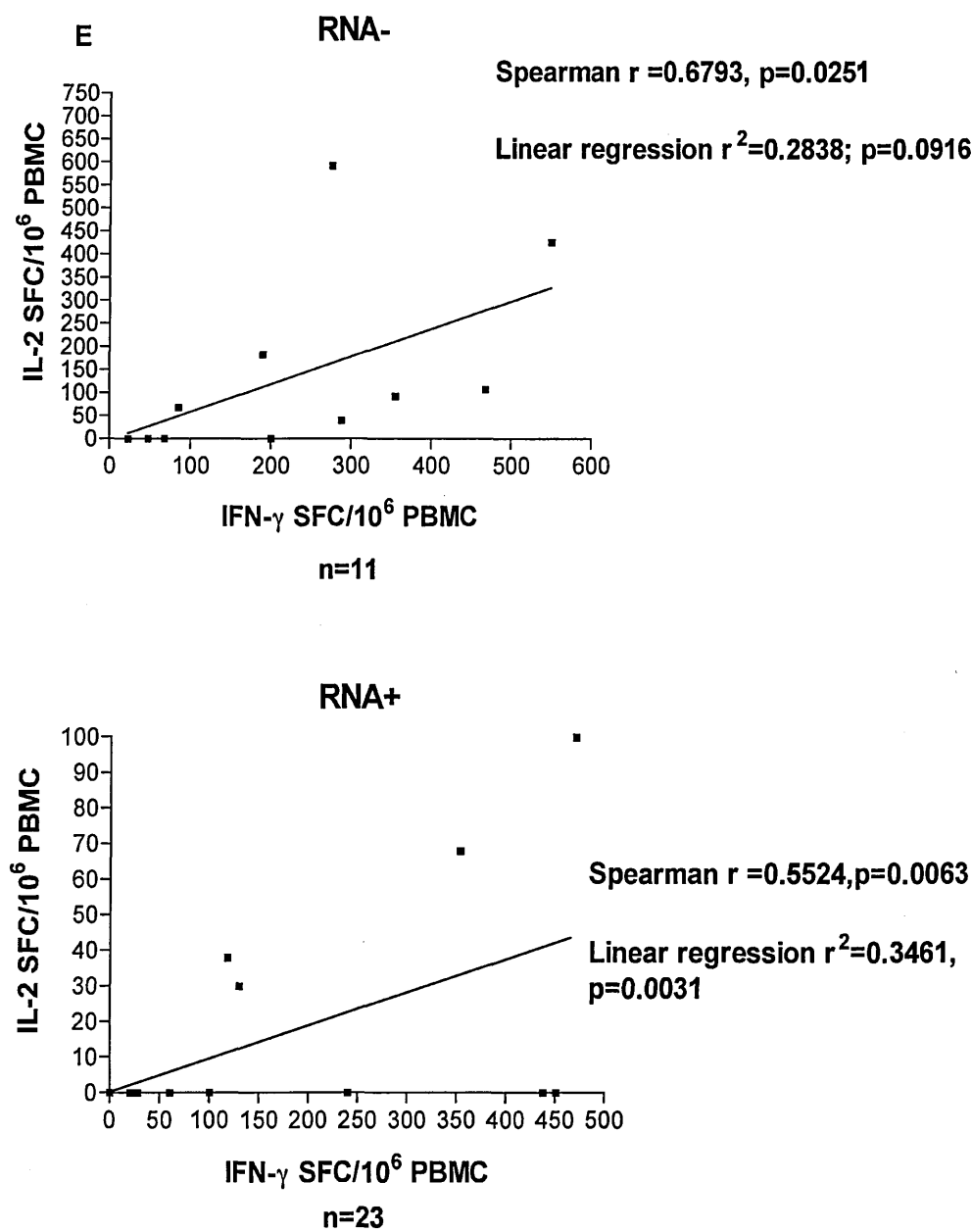


Fig. 4.2D. Relation between viral genotype and HCV-specific T cell responses.

ELISpot IFN- $\gamma$  responses are shown for 7 genotype-3 and 16 genotype non-3 RNA+ individuals. Shown responses are based on those presented in Fig. 4.2.A

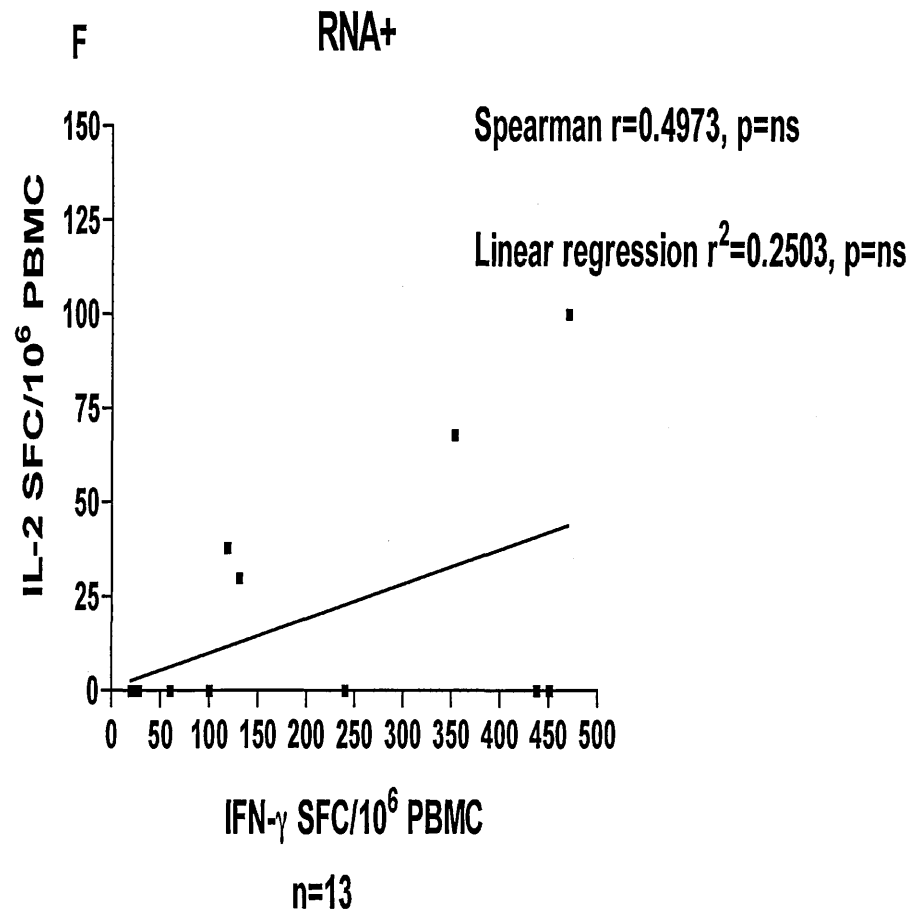
### **3.3.3 Correlation between IL-2 and IFN- $\gamma$ secretion**

I next was interested to see whether there was a correlation between the IL-2 and IFN- $\gamma$  secretion. IL-2 as a growth factor precedes IFN- $\gamma$  production by inducing clonal expansion and proliferation. Once cells have proliferated and are activated they are induced to produce IFN- $\gamma$  as an effector cytokine. Therefore one would expect a correlation between the two cytokines. As expected, in the RNA- group a strong correlation between the two cytokines was seen (Fig. 4.2.E;  $r=0.68$ ,  $p=0.0251$ , Spearman test;). In the RNA+ group, although there was a discrepancy between IL-2 and IFN- $\gamma$  secretion, a correlation was also obtained here (Fig. 4.2.E;  $r=0.55$ ,  $p=0.0063$ , Spearman test). But one has to bear in mind that this might be due to the high number of IL-2/IFN- $\gamma$  double negative values, which go through zero (10/23 RNA+ individuals) and therefore contribute to the correlation. Hence, when the ten individuals with double-negative values were excluded, a correlation was no longer significant (Fig. 4.2.F;  $r=0.497$ ,  $p=ns$ , Spearman test).



**Fig. 4.2.E. Correlation between IL-2 and IFN- $\gamma$  secretion.**

Upper panel shown for 11 RNA- individuals. Lower panel shown for 23 RNA+ individuals. Shown IL-2 and IFN- $\gamma$  responses represent same responses as in Fig. 4.2.A+B. Spots of 10 out of 23 RNA+ individuals go through zero. Spearman test in parallel with the linear regression as an alternative were performed.

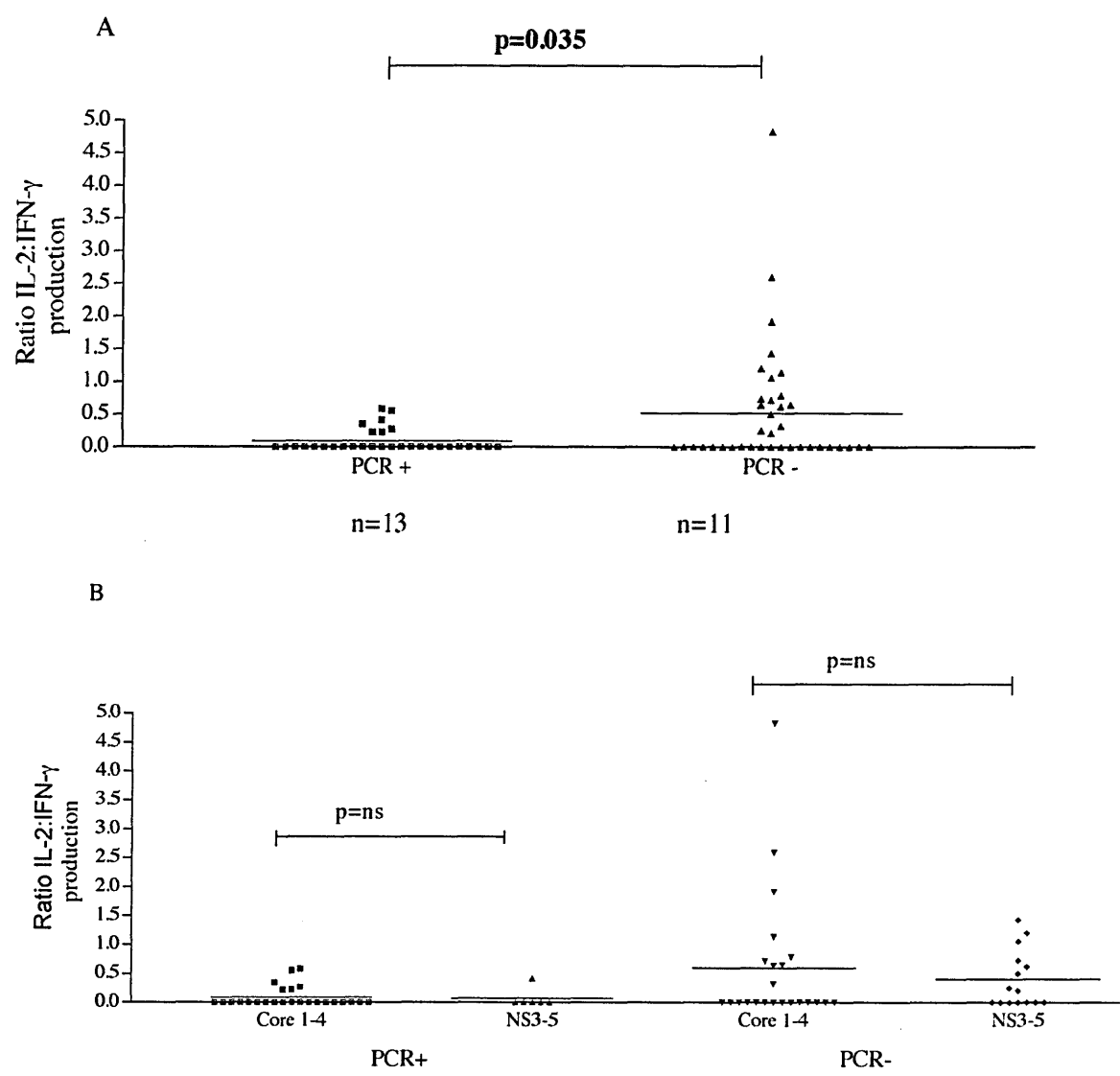


**Fig. 4.2.F. Correlation between IL-2 and IFN-γ secretion.**

Same as for lower panel of Fig. 4.2.E. But here, 10 individuals with IL-2/IFN-γ double-negative responses were excluded. The merged spots between 0 and 50 IFN-γ SFC/10<sup>6</sup> PBMC represent 4 individuals.

**3.3.4. IL-2/IFN- $\gamma$  ratio is significantly reduced in RNA+ group**

Comparing individual antigens (for those antigens where a positive response was obtained), the ratio of IL-2-producing cells to IFN- $\gamma$ -producing cells in the RNA- group was between 1:1 and 1:2, and approximately 1:10 for the RNA+ group (mean 0.5 vs. mean 0.08,  $P=0.035$ , Mann-Whitney test; Fig. 4.3A). Overall, the ratio of total level of IL-2 production (summed across antigens) compared with total IFN- $\gamma$  production was approximately 0.6 in responders and 0.06 in chronically infected persons. The relative deficit in IL-2 production in RNA+ individuals was similar in responses to core peptides and NS3-5 antigens ( $P$  value not significant, Mann-Whitney test; Fig. 4.3B). The IL-2:IFN- $\gamma$  ratios in responses to both core peptides and NS3-5 proteins remained lower in RNA+ individuals than in RNA- negative individuals when tested separately ( $P=0.03$  and  $P=0.04$ , respectively; Mann-Whitney).



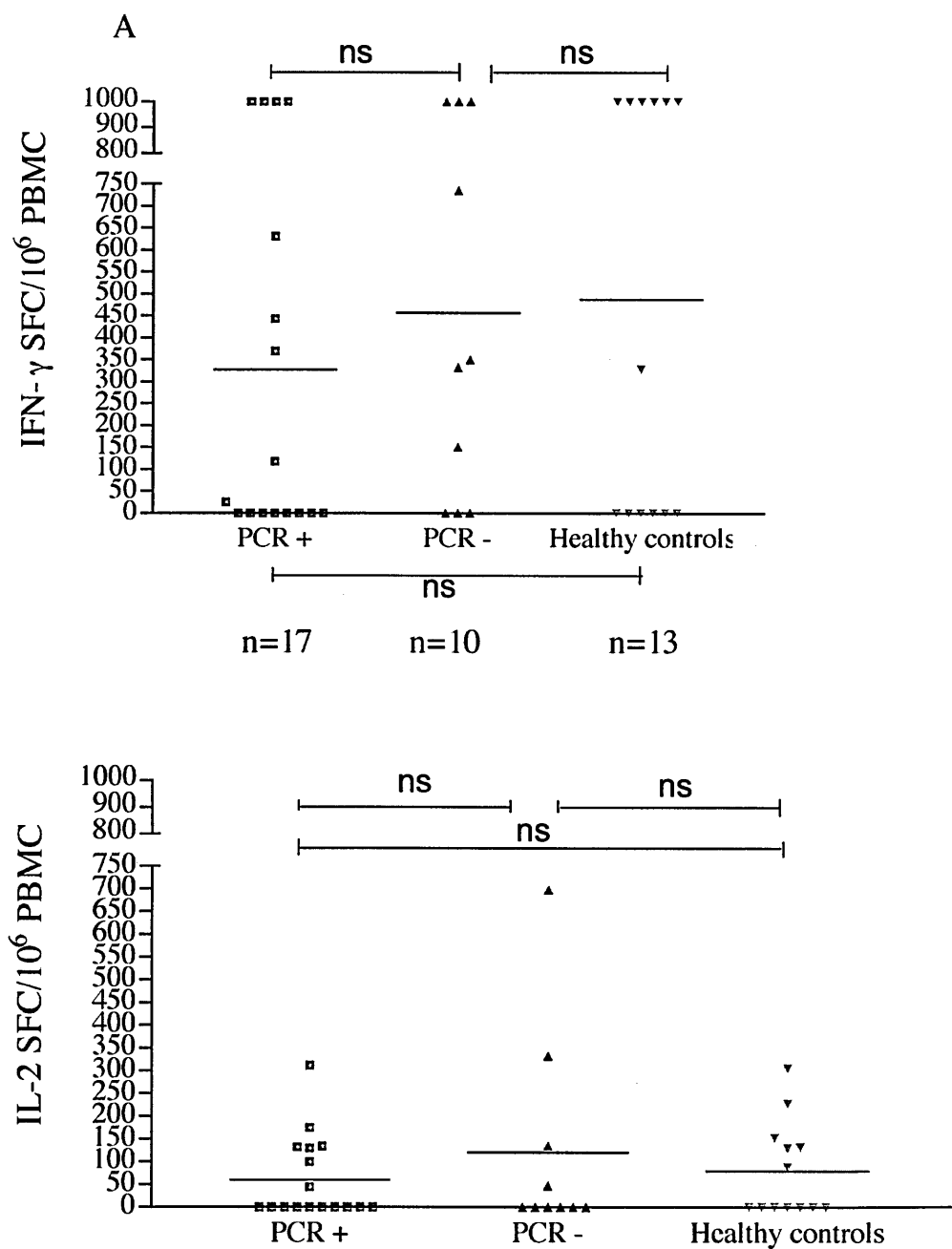
**Figure 4.3.A+B. Ratio of IL-2 to IFN- $\gamma$  producing T cells in the ELISpot.**

A, Ratio of IL-2 to IFN- $\gamma$  production shown for each individual antigen (HCV core peptide pools 1, 2, 3, 4 and non-structural proteins NS3, NS3/4, NS4 and NS5) in both RNA+ and RNA- groups (only those where at least one cytokine was positive were included). B, Ratio of IL-2 to IFN- $\gamma$  production shown for each individual antigen by separation of the core peptide pools 1-4 and the non-structural proteins NS3-5. PCR+ = RNA+; PCR- = RNA- .

### 3.4. Lack of IL-2 production in RNA+ individuals is a specific feature of HCV

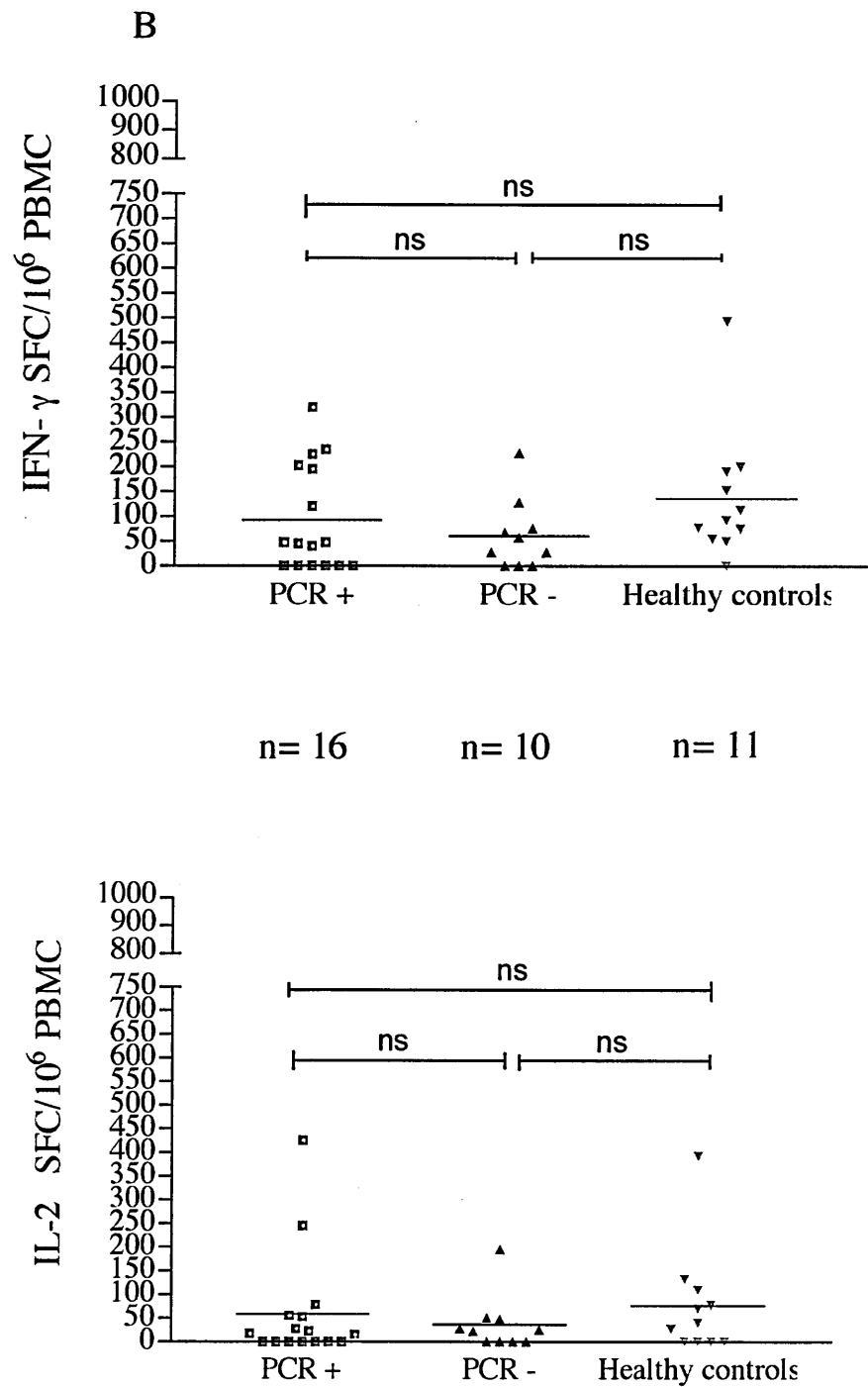
To determine whether the weak IL-2 responses seen in patients with chronic HCV infection was a characteristic of HCV-specific cells only, responses to cytomegalovirus, a persistent virus associated with expanded pools of effector T cell memory, and tetanus toxoid, a non-persistent protein antigen associated with resting central T cell memory, were analysed. The overall magnitude of IFN- $\gamma$  and IL-2 responses to cytomegalovirus did not differ between seronegative healthy controls, RNA+ and RNA- negative groups (Fig. 4.4A; Mann-Whitney test). A strong IFN- $\gamma$  response compared with the IL-2 response was seen in each group, with a ratio of approximately 1 IL-2 secreting cell to 5 IFN- $\gamma$  secreting cells in each case ( $P=0.005$ ,  $P=0.01$  and  $P=0.005$  for healthy controls, RNA- and RNA+ groups, respectively; paired t test). The tetanus toxoid responses were generally weaker and more comparable to those against HCV, but no significant differences were observed between the groups (Fig. 4.4B; Mann-Whitney test). The ratio of IL-2 to IFN- $\gamma$  secreting cells for the tetanus toxoid-specific response was higher than for cytomegalovirus, between 1:1 and 1:2 ( $P=0.02$ ,  $P$  value not significant,  $P$  value not significant for healthy controls, RNA- and RNA+ groups, respectively; paired t test). Thus responses against HCV were weaker in the RNA+ group, but the most striking finding overall was a lack of IL-2 secretion rather than a lack of IFN- $\gamma$  secretion, especially against the core peptide pools.





**Figure 4.4.A. Overall magnitude of IFN- $\gamma$  and IL-2 responses to CMV as determined by ELISpot.**

CMV responses for IFN- $\gamma$  (upper panel) and IL-2 (lower panel) tested in the RNA+, RNA- and healthy controls group. Differences remained non-significant even after very high responses ( $>1000$  IFN- $\gamma$  SFC/ $10^6$  PBMC) were excluded. Not all individuals were tested against CMV (see also table 4.2). PCR+ = RNA+; PCR- = RNA- .



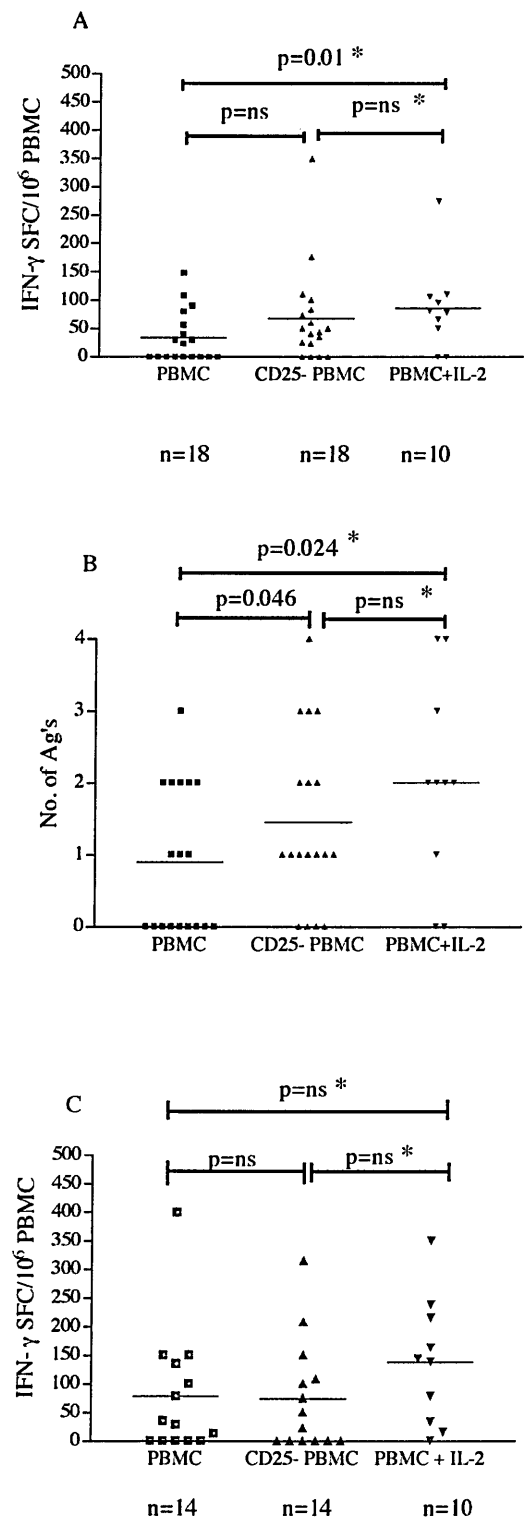
**Figure 4.4.B. Overall magnitude of IFN- $\gamma$  and IL-2 responses to TT as determined by ELISpot**

TT responses for IFN- $\gamma$  (upper panel) and IL-2 (lower panel) tested in the RNA+, RNA- and healthy controls group. Not all individuals were tested against TT (see also table 4.2).

### 3.5. Restoration of HCV-specific CD4<sup>+</sup> T cell responsiveness by exogenous IL-2 addition

IL-2 alone is able to stimulate IFN- $\gamma$  secretion in T cells through activation of intracellular Jak/Stat pathways (Bream et al., 2004). Therefore, the lack of IL-2 secretion seen in RNA<sup>+</sup> individuals might account for loss of overall functional capacity in HCV specific CD4<sup>+</sup> T cell populations. To determine whether it was possible to restore IFN- $\gamma$  production by HCV-specific CD4<sup>+</sup> T cells *in vitro*, the effect of IL-2 addition was studied. In addition, CD25<sup>+</sup> regulatory CD4<sup>+</sup> T cells have been shown to play important roles in control of a variety of responses against pathogens in both human and in animal models (Lin et al., 2002; Oldenhove et al., 2003; Suri-Payer et al., 1998; Suvas et al., 2003; Woo et al., 2002), and a role for such activity in control of HCV specific CD8<sup>+</sup> T cell responses has been proposed (Sugimoto et al., 2003). CD25<sup>+</sup> T cells may compete for IL-2, thus effectively lowering the concentration available for antigen-specific T cells (de la Rosa et al., 2004). Hence, the effect of CD25<sup>+</sup> cell depletion on IFN- $\gamma$  secretion was also examined.

Whole PBMC, CD25<sup>+</sup> cell-depleted PBMC, and PBMC in the presence of additional recombinant IL-2 were tested for HCV responsiveness in IFN- $\gamma$  ELISpot assays. Figure 4.5A demonstrates the restoration of immune responsiveness *in vitro* through the addition of IL-2. Interestingly, IL-2 not only restored the magnitude of responses, but also revealed new responses – thus increasing the breadth of the responses detected in the ELISpot assay (Fig. 4.5B).

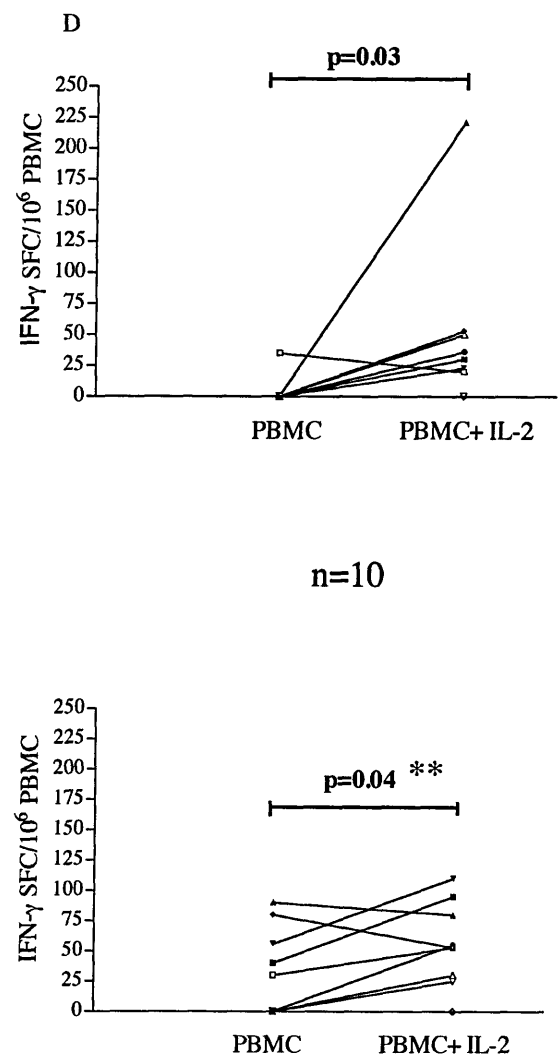


**Figure 4.5.A-C. Immunomodulatory effect of IL-2 on IFN- $\gamma$  HCV-specific responses by *in vitro* stimulation.**  
A, HCV-specific IFN- $\gamma$  ELISpot responses in a group of chronically infected HCV patients were tested using whole PBMC,

CD25<sup>+</sup> cell depleted PBMC and PBMC with addition of exogenous IL-2. The dots shown represent the overall magnitude of IFN- $\gamma$  producing T cells in each tested individual. The summed responses were derived by antigens used for the stimulation with core peptide pools 1, 2, 3, 4 and the non-structural proteins NS3, NS3/4, NS4 and NS5. Mean and SD for background in negative controls did not change significantly after IL-2 stimulation. *B*, Breadth of HCV-specific T cell responses represented as the number of positive antigens in the ELISpot (No. of Ag's) shown for the same group and same antigens with whole PBMC, CD25<sup>+</sup> cell depleted PBMC and PBMC with addition of exogenous IL-2. *C*, IFN- $\gamma$  ELISpot for the same chronically infected HCV group tested for TT-specific responses with whole PBMC, CD25<sup>+</sup> cell-depleted PBMC and PBMC with addition of exogenous IL-2.

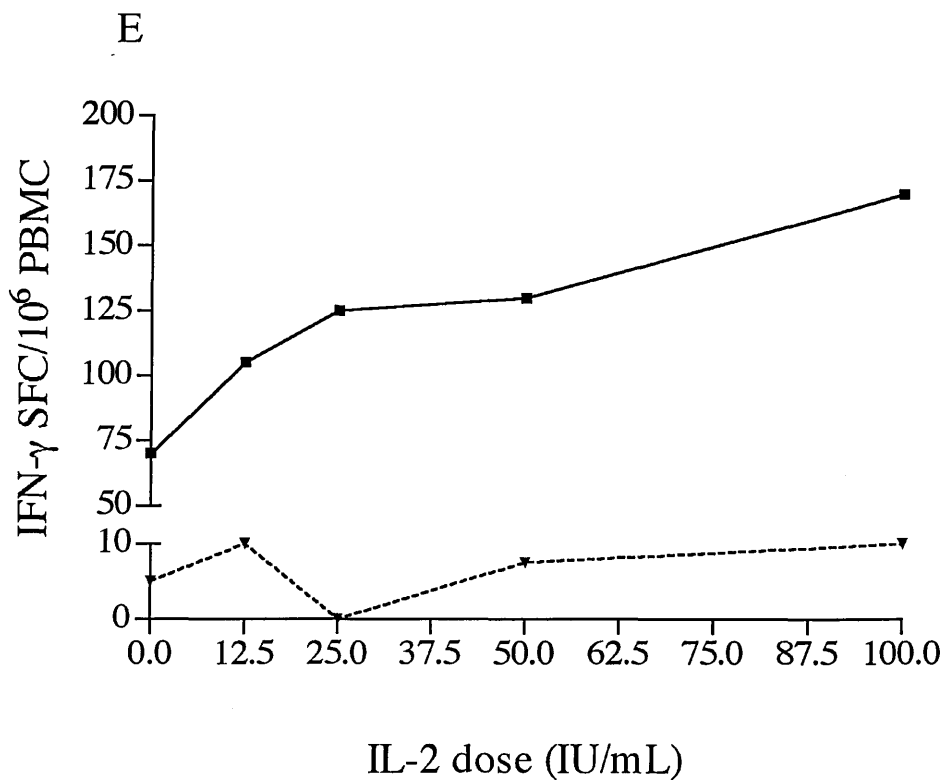
\* paired t-test for comparison of 10 individuals. The unpaired t-test, comparing 18 vs. 10 individuals showed similar results.

Overall, the effect was highly significant when analysis of HCV-specific responses was performed, but not significant for the control tetanus toxoid response. Responses to tetanus toxoid for each tested individual with and without IL-2 treatment are shown in Fig. 4.5C. The addition of IL-15 *in vitro* led to major increases in background IFN- $\gamma$  release, thus making analysis of specific T cell populations impossible (data not shown). Depletion of CD25<sup>+</sup> cells, which includes CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells as well as activated T cells, had a less marked effect on the magnitude and breadth of HCV-specific responses (Fig. 4.5A+B). There was no significant effect on control responses in these individuals (Fig. 4.5C). The increase in responsiveness to HCV antigens was observed both against NS proteins and core peptides, although most clearly in the former (Fig. 4.5D). The effect of IL-2 was dose-dependent in the presence of antigens and not significant in their absence (Fig. 4.5E).



**Fig. 4.5.D. Immunomodulatory effect of IL-2 on IFN- $\gamma$  HCV-specific responses by *in vitro* stimulation**

HCV-specific IFN- $\gamma$  ELISpot responses separated into non-structural proteins NS3-5 (upper panel) and core peptide pools 1-4 (lower panel) before and after exogenous IL-2 stimulation. \*\* 1-tailed paired t-test



**Fig. 4.5.E. IL-2 dose titration curve.**

Immunomodulatory effect of IL-2 on IFN- $\gamma$  HCV core peptide pool 1-4 specific responses in one RNA+ individual, shown by a dose titration curve with 4 different doses of IL-2 (12.5 IU/mL, 25 IU/mL, 50 IU/mL and 100 IU/mL). Continuous line: IFN- $\gamma$  responses represent mean magnitude of duplicate wells by stimulation with core peptide pools 1-4 at different IL-2 concentrations. Dashed line: IFN- $\gamma$  responses of the negative controls at different IL-2 concentrations, showing no significant increase in the background. Due to the lack of cells titrations could be only performed in 3 RNA+ individuals. Titrations of the other 2 RNA+ individuals showed similar results.

## 4. Discussion

Resolved HCV infection is characterised by relatively strong T cell responses that are detectable for years after initial infection, even after loss of detectable antibody responses (Day et al., 2002; Lauer et al., 2004). In particular, the hallmark of such individuals is the maintenance of strong T cell proliferative responses to a range of antigens. Here I demonstrate that such responses are characterised by strong IL-2 secretion amongst CD4<sup>+</sup> T cell populations. Data using cytokine capture assays with magnetic bead enrichment confirmed the presence of large populations of CD4<sup>+</sup> T cells secreting IL-2, and many IFN- $\gamma$ /IL-2 double positive cells. The ELISpot assays confirmed similar proportions of IL-2- and IFN- $\gamma$ -secreting cells, although these assays cannot verify whether such cells were double-positive for the cytokines tested. By analogy with HIV, these are similar to CD4<sup>+</sup> T cell populations found under conditions when the viral load is low (Younes et al., 2003).

Chronic HCV infection results in a range of clinical severities. The majority of the individuals tested here were found to have low IL-2 secretion. The study was, however, skewed towards those with lower levels of fibrosis. This was because I was interested in testing patients who had not been previously treated, as IFN- $\alpha$  and ribavirin can influence both T cell function and cytokine secretion profiles (Barnes et al., 2002; Barnes et al., 2004a). Additionally, the relationship between IL-2 secretion and viral load was not assessed here. Unfortunately, viral loads were not available to test whether there is a relation between T cell responses and viral load.



However, T cell responses have been shown to correlate inversely with the viral load in chronically HCV-infected patients not only in PBMC (Janvier et al., 2005), but also in the liver (Freeman et al., 2003). This inverse relationship supports the hypothesis that HCV-specific T cell responses limit viral replication in patients with chronic HCV infection. If this is the case, then one could speculate that at least the four RNA+ patients in this study with IFN- $\gamma$  responses  $>225$  SFC/ $10^6$  PBMC have lower viral loads than those with fewer IFN- $\gamma$  responses. Currently, this is just a speculation as, unfortunately, we do not have the viral loads to confirm it.

A prospective study looking at the IL-2 and IFN- $\gamma$  secretion in more severely affected patients over a range of viral loads as well as assessing the potential impact of therapy will be one goal of the future work.

Compared to studies that largely use proliferation assays, a significant fraction of persistently infected individuals did maintain IFN- $\gamma$ -secreting cell populations, but very few were found to secrete IL-2. This is consistent with the low proliferative capacity typically associated with HCV-specific populations during chronic infection, even after therapy (Chang et al., 2001b; Hoofnagle, 2002; Missale et al., 1996; Rahman et al., 2004). Low levels of IL-2 secretion have been shown in independent studies in humans and mice to be accompanied by loss of proliferative capacity both *in vitro* and *in vivo* (Fuller and Zajac, 2003; Iyasere et al., 2003; Wherry et al., 2003a; Younes et al., 2003). Proliferative responses were not assessed in detail in this group.

However, a previous study, using identical antigens in a similarly recruited cohort, showed strong and multispecific proliferative responses in 5/15 spontaneously resolved individuals, but only in 1/21 with untreated persistent infection. The concentration of protein used in my assays (1 µg/mL) is relatively low, which may potentially reduce the frequency of strong responders in proliferation assays. However, the response rates in ELISpots are high as shown here, and a clear difference was seen between the clinical groups exposed to similar antigens and concentrations.

Lack of IL-2 secretion is most readily explained by overstimulation of CD4<sup>+</sup> T cells, which is analogous to the state of T cells in untreated HIV infection (Boaz et al., 2002; Day and Walker, 2003; Younes et al., 2003). As T cell populations proliferate in response to antigen, they lose IL-2 secretory capacity as they acquire effector characteristics. In cases where stimulation is extreme and/or prolonged, loss of cytokine secretion may occur successively, before deletion occurs.

In this case, I expect that reduction in viral load through combination therapy may contribute to the well-documented restoration of CD4<sup>+</sup> T cell responsiveness through a return to an IL-2 secreting state (Barnes et al., 2002; Kamal et al., 2002). This hypothesis will be tested in future longitudinal prospective studies.

A limitation of this and other studies is that only genotype 1 antigens were used. Nevertheless, it was interesting to see that those patients infected with HCV genotype 3 were more likely to display higher IFN-γ T cell responses than those infected with genotype non-3. Evidence for a link between genotype and HCV-specific T cell responses has been shown before (Hultgren et al., 2004).

One explanation for our findings could be that individuals with genotype 3 infection were infected with genotype 1 previously. Recently, it has been reported that the T cell response to a previously cleared infection with genotype 1 may remain detectable despite the fact that the patient was subsequently infected with genotype 3 (Harcourt et al., 2003). On the other hand, cross-reactive responses may explain the high frequencies of genotype 3 responses as the core protein sequences of genotype 1 and 3 are relatively conserved. But overall, I feel that genotype-specific antigens and sequencing of the epitopes concerned are needed to fully address these issues.

Interestingly, *in vitro* IL-2 treatment was able to markedly influence IFN- $\gamma$  responsiveness of HCV-specific CD4<sup>+</sup> T cell populations. The depletion of CD25<sup>+</sup> cells had a modest influence on IFN- $\gamma$  responsiveness in these assays (Camara et al., 2003). However, I only performed simple CD25<sup>+</sup> T cell depletion, which may remove activated T cells in addition to other populations that are not CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells. Additionally, differences in the bead preparation used may also influence the level of CD25 expression on T cells that are depleted. However, despite these limitations, I used this depletion as a simple method to remove cells expressing high levels of the IL-2 receptor, which might then lead to the “release” of IL-2 for use by antigen-specific cells (de la Rosa et al., 2004).

The effect of adding IL-2 *in vitro* was obvious both in terms of response breadth and magnitude. The effect was clear-cut on HCV-specific T cell populations only, although it is known that IL-2 can restore T cell responsiveness in other disease settings such as HIV infection (Marchetti et al., 2004; Paiardini et al., 2001).

The mechanism is likely to be a direct action on T cells, although in such assays, an indirect effect through other T or non-T cell populations cannot be excluded.

These findings contrast in some ways with the limited comparison previously made using MHC class II tetramers (Day et al., 2003). There, a set of three tetramers containing NS3/4 derived peptides and a single MHC class II molecule (DRB1\*0401) were used to study small groups of RNA<sup>+</sup> and RNA<sup>-</sup> individuals. While tetramer positive cells were detectable in those with resolved infection, no responses were detectable in 4 individuals with persistent viraemia. In this chapter, responses to non-structural proteins were rarely detected in RNA<sup>+</sup> individuals; however, overall, responses are still detectable, particularly to core epitopes. It is possible that some CD4<sup>+</sup> T cell responses are indeed lost, through escape mutations or exhaustion, whilst others are maintained- perhaps those of lower avidity. The fine mapping and restriction of IFN- $\gamma$ <sup>+</sup>/IL-2<sup>-</sup> responses in RNA<sup>+</sup> individuals will allow in future further detailed comparisons using novel MHC class II tetramer reagents. One important bonus of MHC class II tetramers is the capacity to examine the surface and intracellular phenotype of antigen-specific CD4<sup>+</sup> T cells *ex vivo*. In previous studies it has been observed that HCV-specific memory populations are typically CD62L and CCR7 high (i.e., have features of “central” memory T cells) (Day et al., 2003). While examination of phenotype is theoretically possible using flow cytometric cytokine assays, even short-term stimulation can modify markedly the surface expression of CD62L and CCR7, making them unsuitable for this purpose. Thus, in this chapter, I have few data on the status of the antigen-specific T cells in this cohort of RNA<sup>+</sup> patients.

A recent study of HIV-specific CD4<sup>+</sup> T cells using intracellular cytokine staining, which are typically IL-2 low/IFN- $\gamma$  high in untreated infection (Younes et al., 2003), revealed these to be CCR7 low and CD62L low, with CCR7 expression correlating inversely with viral load (Scriba et al.).

The responses identified here are defined as CD4<sup>+</sup> T cell responses through a combination of depletion experiments and confirmation by flow cytometric analysis. CD8<sup>+</sup> T cell responses have been extensively examined by our group and others (Lauer et al., 2004; Wedemeyer et al., 2002). Interestingly, very few responses to overlapping core peptides were observed, using a comprehensive screening technique, especially in RNA<sup>+</sup> patients (0/20). In future studies of the function of HCV-specific CD8<sup>+</sup> T cell populations, it will be important to assess IL-2 as well as IFN- $\gamma$  secretion.

In practical terms, the analysis of CD4<sup>+</sup> T cells through IL-2 ELISpots is simple and robust and gives important information as to the functional activity of specific T cell populations. As shown in figure 1A+B, it is also possible to identify these cells using flow cytometric techniques, but these require magnetic bead enrichment in order to gain accurate staining *ex vivo* (Barnes et al., 2004b; Day et al., 2003). The sensitivity for IL-2 secretion using this method seemed to be slightly higher than that of the ELISpot, although the ELISpot technique is directly quantitative without enrichment and much more suited for clinical studies using a range of antigens. *In vitro* IL-2 treatment reveals *de novo* responses in some chronic patients.

These responses may potentially be restored through conventional therapy or immunomodulation. Finally, the fact that such T cells are intact and can respond to IL-2 *in vitro* indicates that cytokine treatment *in vivo* may be beneficial.

## 5. Conclusions

1. Persistent HCV infection is associated with an IFN- $\gamma^+$ /IL-2 $^-$  population of CD4 $^+$  T cells.
2. These cells seen are primarily directed against core-derived peptides.
3. The functionality of these cells can be restored with *in vitro* IL-2 administration.
4. Modest restorative effects can be seen using depletion of CD25 $^+$  cells

In the next chapter, I will study similar groups to further evaluate the functionality and the phenotype of HCV-specific CD4 $^+$  T cells in persistent and resolved infection. In more detail, I will be comparing the IL-2/IFN- $\gamma$  cytokine secretion with the CD4 $^+$  T cell proliferative capacity to see whether there is a difference between persistent and resolved infection. In chapter 5, of the newly recruited RNA $^+$  patients only one is from chapter 4 (see Table 4.2). Eight of the 12 RNA $^-$  individuals in the following chapter were used in chapter 4 (Table 4.2). But in the following study assays have been newly set up for those as well.

## CHAPTER 5

### LINKING CYTOKINE SECRETING CAPACITY AND PROLIFERATIVE CAPACITY OF CD4<sup>+</sup> T CELLS IN PERSISTENT AND RESOLVED HCV INFECTION

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## 1. Abstract

**Background:** CD4<sup>+</sup> T cell responses are important for the outcome of HCV infection. However, the functional status of HCV-specific CD4<sup>+</sup> T cells is poorly understood. We used a set of *ex vivo* assays to evaluate the functionality and phenotype of HCV-specific CD4<sup>+</sup> T cells in persistent and resolved infection.

**Methods:** PBMC from 24 prospectively recruited HCV RNA<sup>+</sup> individuals, 12 spontaneously resolved individuals (i.e. HCV Ab<sup>+</sup>, RNA<sup>-</sup>) and 11 healthy controls were analysed for IFN- $\gamma$  and IL-2 secretion by ELISpot. HCV-specific CD4<sup>+</sup> proliferative responses of CFSE-labelled PBMC were assessed using a single cell flow cytometric assay after 6 days of stimulation. HCV core-derived peptides (20 mers overlapping by 10) spanning aa 1-191 and recombinant proteins NS3/4 and NS5 were used for both ELISpot and CFSE assays.

**Findings:** As demonstrated in chapter 4, sustained responses to core-derived peptides were observed in the RNA<sup>+</sup> group. However, using the sensitive CFSE assay, proliferation of HCV-specific CD4<sup>+</sup> T cells in the RNA<sup>+</sup> group was substantially reduced on a per cell basis, in parallel to IL-2 secretion, when compared to responses to the same antigens in the RNA<sup>-</sup> group. Depletion of CD62L<sup>+</sup> cells in the IFN- $\gamma$  ELISpot indicated that these IFN- $\gamma$ <sup>+</sup> proliferation<sup>low</sup> CD4<sup>+</sup> cells include CD62L<sup>low</sup> “effector” memory cells. In RNA<sup>-</sup> individuals, a strong relationship between cytokine secretion and proliferative capacity was seen. However, in RNA<sup>+</sup> individuals, IFN- $\gamma$  secretion far exceeded proliferative capacity.

**Conclusions:** During persistent HCV infection, some CD4<sup>+</sup> T cell specificities appear to be lost, as measured using a range of techniques, but others are maintained, apparently as “effector memory” populations. Such subsets may yet play a significant role *in vivo* and also provide a substrate for modulation in immunotherapeutic interventions.

## 2. Introduction

The mechanisms behind HCV persistence are poorly understood. Once persistent infection is established, in most studies CD4<sup>+</sup> T cell responses in blood appear to be weak, as compared with those individuals who spontaneously resolve infection (Day et al., 2002; Ferrari et al., 1994; Thimme et al., 2001). In particular, chronic infection has been associated with loss of proliferative populations of CD4<sup>+</sup> T cells (Chang et al., 2001b; Ulsenheimer et al., 2003; Wedemeyer et al., 2002). The mechanism behind this is so far unexplained, but since CD4<sup>+</sup> T cells play a critical role in this infection, understanding this phenomenon is of central importance.

It has generally been assumed that loss of proliferation equates to loss of the specific T cell populations. Low levels of IL-2 secretion have been shown in independent studies in humans and mice to be accompanied by a loss of proliferative capacity both *in vitro* and *in vivo* (Fuller and Zajac, 2003; Iyasere et al., 2003; Wherry et al., 2003a; Younes et al., 2003). In HIV infection, recent data suggest that, in the presence of high viral load, HIV-specific CD4<sup>+</sup> T cell populations do exist but lack proliferative capacity (Younes et al., 2003). This status is associated with the production of IFN- $\gamma$  upon antigen stimulation, but little or no IL-2 is expressed. As described in chapter 4, we have shown maintenance of IFN- $\gamma$  secretion with weak IL-2 production by CD4<sup>+</sup> T cells in the previous cohort of chronically HCV-infected patients (Semmo et al., 2005b). Addition of exogenous IL-2 resulted in an increase of IFN- $\gamma$  production in those who lack this cytokine.

The majority of HCV studies which assess CD4<sup>+</sup> T cell proliferation use <sup>3</sup>H-thymidine incorporation assays. However, this technique is relatively insensitive and does not provide information on the phenotype of the proliferating cells in heterogeneous cell populations, such as peripheral blood mononuclear cells (PBMC). Furthermore, because <sup>3</sup>H-thymidine incorporation measures DNA synthesis during a short pulse at the end of a 3-7-day culture period, the fate of the cells that have proliferated cannot be determined. Therefore, we have refined an alternative assay that allows detection, identification and quantification of T cells that proliferate *in vitro*, based on the dilution of the fluorescent dye 5,6-carboxyfluorescein diacetate ester (CFSE) that the cells are prelabelled with. Here we studied a completely new cohort of chronically infected HCV patients and compared their cytokine secretion patterns to their proliferative capacity in parallel with a group that spontaneously resolved HCV. We further aimed to use this assay in combination with *ex vivo* ELISpots to define the origin of the defect of CD4<sup>+</sup> T cell proliferation associated with persistent HCV infection.

### 3. Results

#### 3.1. Study subjects

For the analysis of HCV-specific T cell responses, blood was obtained from 24 untreated patients (Table 5.1; CH1- CH24) with persistent HCV infection (RNA+) and from 12 individuals (R1- R12) with spontaneously resolved HCV infection (RNA-). Eleven healthy individuals from my lab were also recruited as negative controls (healthy controls; age range 24-52). Liver Biopsies on RNA+ patients were scored using the method of Ishak, to assess fibrosis (range 0-6) and inflammation (Ishak et al., 1995).

For detailed methods see methods chapter 2.

Previously, the capacity of virus-specific CD4<sup>+</sup> T cells to secrete cytokines was compared in cohorts of spontaneously resolved and persistently infected HCV patients (Chapter 4) (Semmo et al., 2005b). In this study I tried to link the cytokine secretion with the proliferative capacity of HCV-specific CD4<sup>+</sup> T cells in a completely new cohort of RNA+ individuals, except the patient with the ID CH4 (in chapter 4 with the ID CH21).

Subject	Age	Sex	Staging (0-6)*	Grading (0-18)*	ALT (normal <45 U//L)	Genotype
CH1	43	M	-	-	91	1b
CH2	48	M	-	-	38	2b
CH3	46	M	2		67	1b
CH4	51	M	6	n.a.	281	3
CH5	63	M	1	4	43	1
CH6	35	M	1	5	181	4
CH7	42	M	2	6	79	1
CH8	34	M	1	4	91	3a
CH9	56	M	6	8	277	1
CH10	22	F	-	-	33	2b
CH11	34	F	1	4	40	1a
CH12	30	M	-	-	329	1a
CH13	41	M	-	-	32	1b
CH14	25	M	-	-	114	3a
CH15	42	F	0	2	34	3a
CH16	43	F	-	-	55	1
CH17	46	M	1	1	17	4c/d
CH18	67	M	-	-	118	1
CH19	66	M	-	-	231	-
CH20	66	F	1	2	18	1
CH21	40	F	0	2	63	1a
CH22	32	M	0	2	141	1b
CH23	57	M	2	5	59	1a
CH24	39	M	-	-	80	3
R1	58	M				
R2	44	M				
R3	76	F				
R4	45	F				
R5	54	M				
R6	42	F				
R7	48	M				
R8	47	F				
R9	69	F				
R10	43	F				
R11	41	M				
R12	50	F				

Table 5.1. Baseline characteristics of the patients

ALT= Alanine Aminotransferase; \*Staging and grading was clinically scored according to Ishak’s Scoring method (Ishak et al., 1995); -, no biopsy performed; For RNA-group ALT levels were previously tested normal. Viral load for RNA+ group was not available.

A	B	C	D	E	F	G	H	I
	CFSE Assay		ELISpot Assay					
ID	P1-4	NS3-5	P1-4 IFN- $\gamma$	NS3-5 IFN- $\gamma$	P1-4+NS3-5 IFN- $\gamma$	P1-4 IL-2	NS3-5 IL-2	P1-4+NS3-5 IL-2
CH1	0	0	83	0	83	38	0	38
CH2	1	11	405	268	673	158	88	246
CH3	0	0	40	0	40	0	0	0
CH4	0	0	85	0	85	55	0	55
CH5	0,5	0	0	0	0	0	0	0
CH6	0,7	0	63	0	63	0	0	0
CH7	0,5	0	155	0	155	40	0	40
CH8	8,4	1,9	45	0	45	23	0	23
CH9	1,6	1,5	108	0	108	75	0	75
CH10	1,4	0	65	0	65	0	0	0
CH11	3,3	0	0	0	0	38	0	38
CH12	0,9	0	20	0	20	0	0	0
CH13	15,5	0,9	65	60	125	48	30	78
CH14	0	0	0	0	0	0	0	0
CH15	0	0	-			15	0	15
CH16	0	0				63	0	63
CH17	0	0				25	0	25
CH18	0,26	0	0	0	0	0	0	0
CH19	0	0	93	0	93	0	0	0
CH20	1,28	0	213	0	213	83	0	83
CH21	0,09	0	43	0	43	18	0	18
CH22	0	0	85	0	85	0	0	0
CH23	0,34	0	0	0	0	0	0	0
CH24	0	0	0	0	0	0	0	0
R1	0,4	14,2	18	0	18	18	0	18
R2	1,1	1,4	28	0	28	63	0	63
R3	29	2,3	175	38	213	477	40	517
R4	32	21,35	255	155	410	115	60	175
R5	0	40,43	0	235	235	0	50	50
R6	4,2	18,23	0	155	155	20	90	110
R7	0	0	0	50	50	0	25	25
R8	0	10,29	123	165	288	0	40	40
R9	0	3,06	*					
R10	6,8	9,46						
R11	*		375	0	375	100	0	100
R12			550	0	550	426	0	426

Table 5.2. CFSE and ELISpot raw data of the patients

Data shown for individuals with persistent and resolved infection. Columns B+C, results of CFSE assays shown for core peptide pools 1-4 (B) and NS3-5 (C). Responses represent the percentage of proliferating CD4<sup>+</sup> T cells. Columns D-I, results of IFN- $\gamma$ /IL-2 ELISpot assays shown for core peptide pools 1-4 and NS3-5. Results shown as SFC/10<sup>6</sup> PBMC.

Column D, IFN- $\gamma$  responses for P1-4; column E, IFN- $\gamma$  responses for NS3-5; column F, IFN- $\gamma$  responses for P1-4 and NS3-5 summed; column G, same as for columns D-F, but here shown for IL-2.

- = assay did not work; \*= few cells, therefore assays could not be performed.

## **3.2. *Ex vivo* cytokine release in spontaneously resolved (RNA-) and chronically infected (RNA+) individuals**

### **3.2.1. *HCV-specific IFN- $\gamma$ production in the ELISpot***

Firstly, PBMCs isolated from individuals with spontaneously resolved (RNA-) and chronic HCV infection (RNA+) were stimulated with HCV antigens, and their ability to secrete IFN- $\gamma$  and IL-2 was analysed using ELISpot assays to assess responses to core, NS3/4 and NS5. Previous depletions of CD8<sup>+</sup> cells from PBMC confirmed that responses are mainly CD4-derived (Chapter4) (Semmo et al., 2005b).

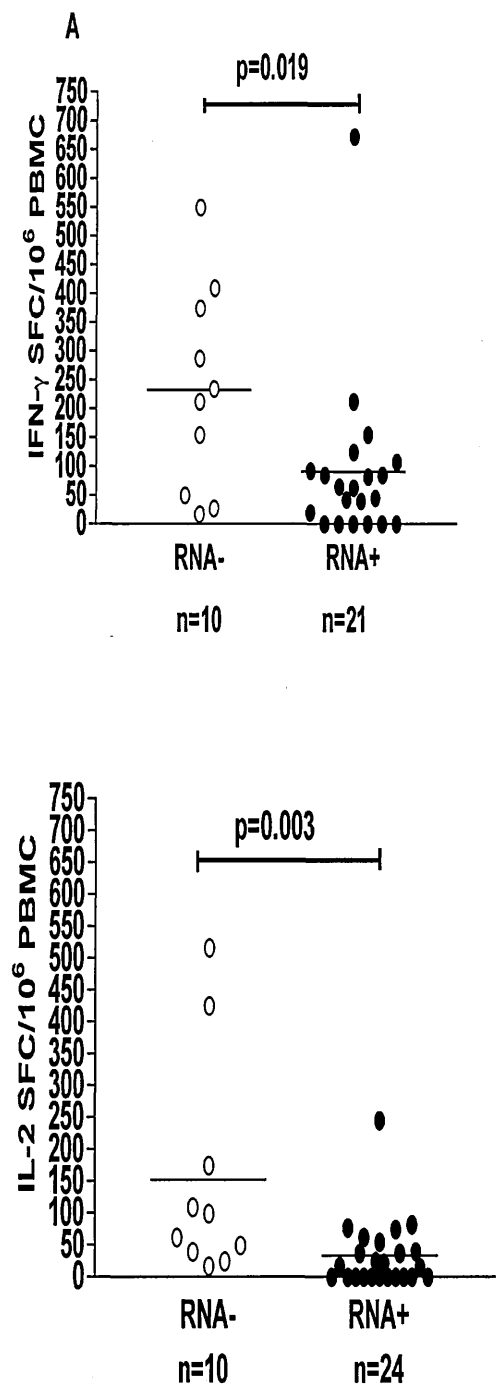
Overall, significantly larger IFN- $\gamma$  ELISpot responses were obtained in the RNA- group than the RNA+ group (Fig. 5.1A). The response difference across all tested antigens, although significant, was only about 2.6-fold (mean 232 spots per million vs. mean 90,3 spots per million;  $P=0.019$ , Fig. 5.1A; Mann-Whitney test). A positive IFN- $\gamma$  response was detected in all (10/10) RNA- individuals, but only in 15/21 RNA+ individuals ( $P=0.14$ ; Fisher's exact test). These data are consistent with previous analyses indicating weaker CD4<sup>+</sup> T cell responses in RNA+ individuals using ELISpot and proliferative assays.



**3.2.2. HCV-specific IL-2 production in the ELISpot**

For the IL-2 ELISpot assays, in the RNA- group, responses across all antigens were similar in overall magnitude to IFN- $\gamma$ ; however, in the RNA+ group, only weak responses were obtained (mean 153 spots per million vs. mean 33,2 spots per million;  $P=0.003$ , Fig. 5.1A; Mann-Whitney test).

A positive IL-2 response was again detected in all RNA- individuals (10/10), but only in 13/24 RNA+ individuals ( $P=0.0135$ ; Fisher's exact test).

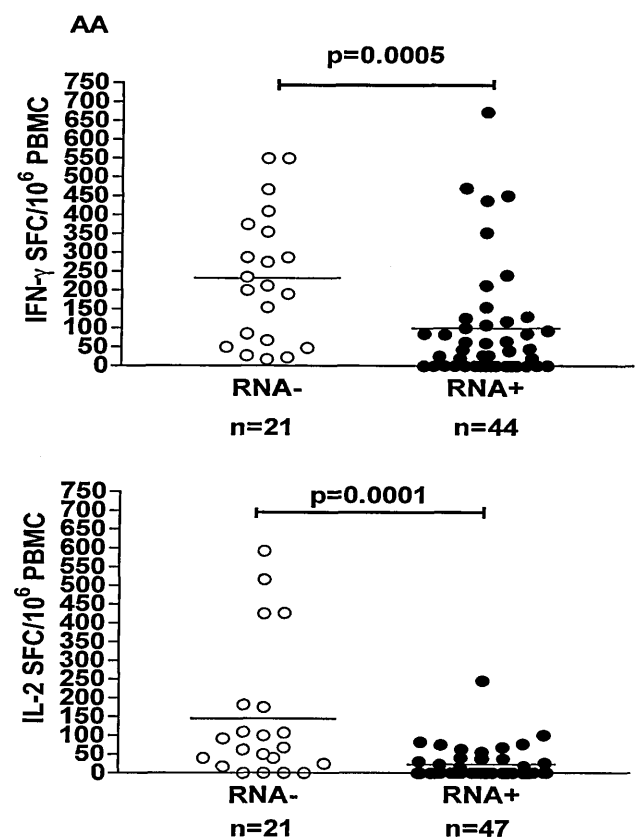


**Fig. 5.1.A. Magnitude of HCV-specific T cell responses as determined by ELISpot for IFN-γ and IL-2.**

PBMC were stimulated using HCV core peptide pools 1-4 or HCV non-structural proteins NS3-S5 as described in the methods. Overall responses of IFN-γ (upper panel) and IL-2 (lower panel) producing PBMC comparing 24 chronic HCV patients (RNA+) with 10 HCV resolvers (RNA-).

Responses represent the mean of duplicate wells after subtraction of the background and responses to core peptides and NS proteins are summed for each individual (one spot for each patient; see also Table 5.2, columns F+I).

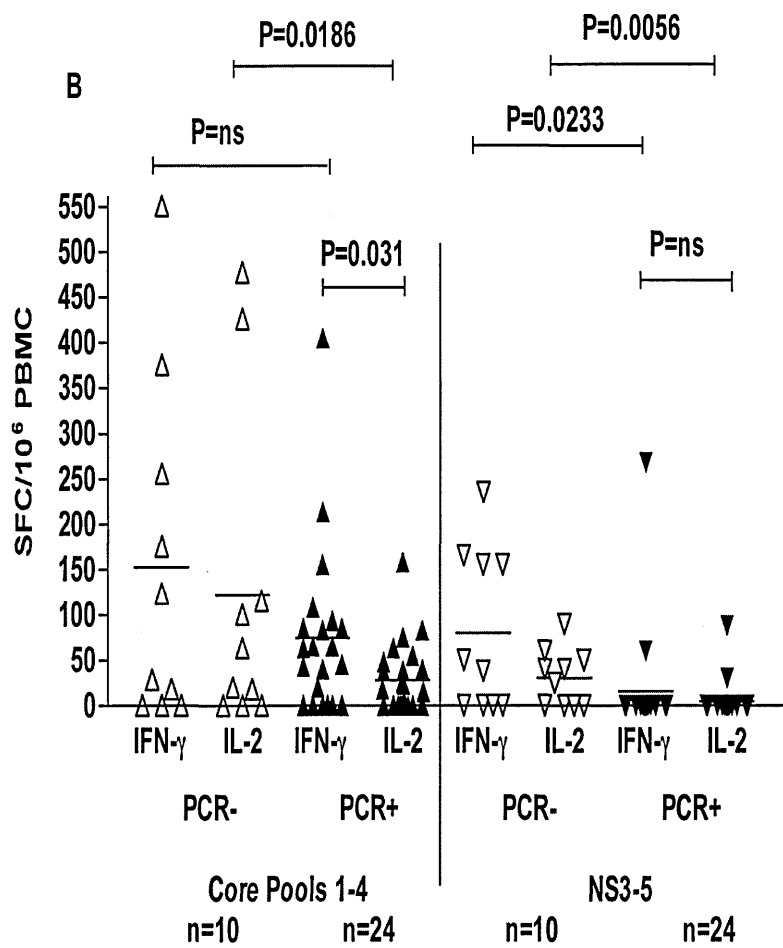
When IFN- $\gamma$  and IL-2 ELISpot responses from this chapter were analysed with those from Fig. 4.2A+B of chapter 4, the significant differences between the two groups were even stronger, as shown here in Fig. 5.1AA.



**Fig. 5.1.AA. Magnitude of HCV-specific T cell IFN- $\gamma$  and IL-2 ELISpot responses.**

As shown in Fig. 5.1.A. But here, results from chapter 5 are put together with those of chapter 4. P values were calculated using Mann-Whitney test.

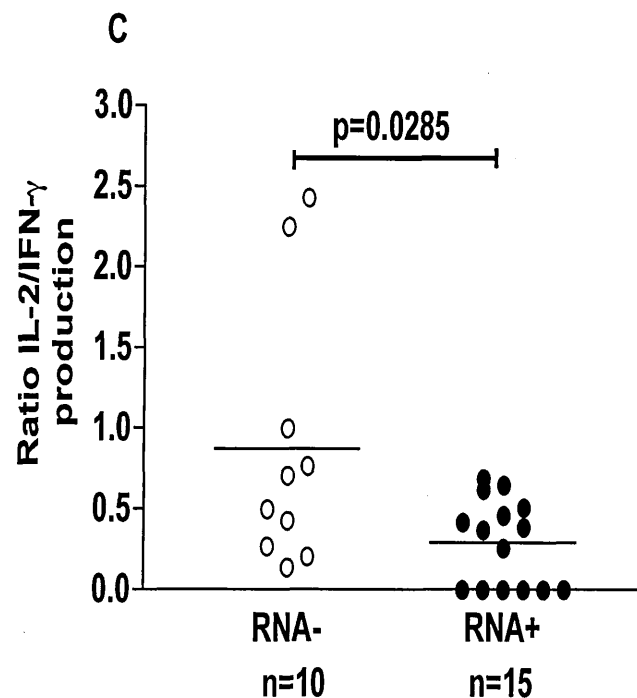
When the responses to different antigens were analysed, the differences between the groups were maintained (Fig. 5.1B). RNA- individuals maintained a broad response detectable against all antigens tested for both IFN- $\gamma$  and IL-2 (P value not significant). As shown before, IFN- $\gamma$  responses against core peptides were common in the RNA+ group, but these were weaker using the IL-2 assay (P=0.0317). The IFN- $\gamma$  responses in the RNA+ group against NS3-5 were less common than those against core peptide pools (2/21 vs. 15/24, P=0.0005; Fisher's exact test).



**Fig. 5.1.B.** As for fig. 5.1.A, but responses of core peptides and NS proteins are displayed separately. Number of RNA+ individuals for IFN- $\gamma$  NS3-5 responses are 21. PCR+ = RNA+; PCR- = RNA- .

### 3.2.3. Reduced IL-2/IFN- $\gamma$ ratio in the RNA+ group

Overall, the ratio of IL-2-producing cells to IFN- $\gamma$ -producing cells was substantially reduced in the RNA+ group compared to the RNA- group (mean 0.3 vs. mean 0.9;  $P=0.0285$ ; Mann-Whitney test) (Fig. 5.1C). In summary, IFN- $\gamma^+$ /IL-2 $^+$  HCV-specific CD4 $^+$  T cell populations were largely detectable in the spontaneous resolvers, while IFN- $\gamma^+$ /IL-2 $^-$  HCV-specific CD4 $^+$  T cell populations predominated in the RNA+ group, validating previous findings (Chapter 4) (Semmo et al., 2005b).

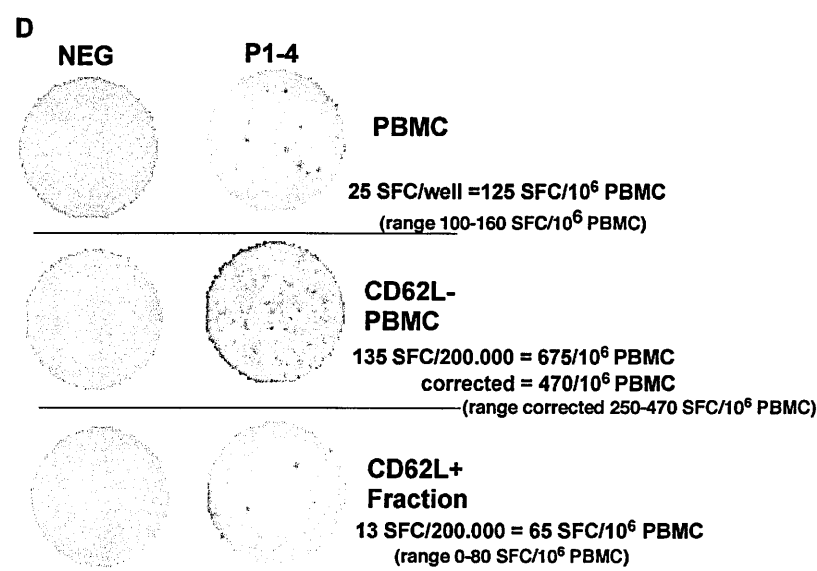


**Fig. 5.1.C. Ratio of IL-2 to IFN- $\gamma$  producing T cells in the ELISpot.**

Shown for HCV core peptide pools 1-4 and non-structural proteins NS3-5 together in both RNA- and RNA+ groups (only those where a response to at least one cytokine was positive were included). The mean ratio for core-specific responses was 1.19 in the RNA- and 0.3 in the RNA+ group (P= .0031).

**3.2.4. Cytokine responses in RNA+ group are mainly “effector memory” cells**

Furthermore, when CD62L depletions were performed in RNA+ individuals, IFN- $\gamma$  responses were maintained in the CD62L-negative PBMC (Figure 5.1D), whereas responses were low or undetectable in CD62L-positive populations. These data suggest that the CD62L<sup>lo</sup> cells comprise a substantial fraction of the responding population, thus representing features of “effector” memory pools.



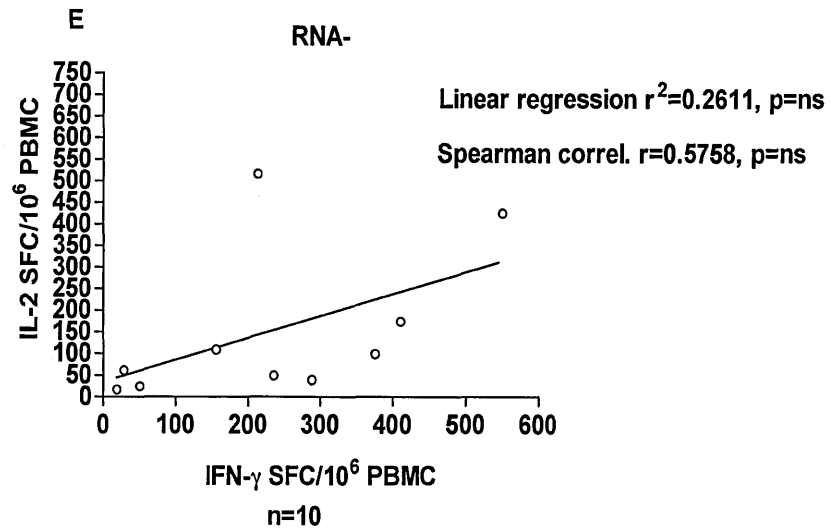
**Fig. 5.1.D. Effector memory cells in RNA+ individuals determined by ELISpot using CD62L depletions.**

The panel shows an IFN- $\gamma$  ELISpot against medium alone and the core peptide pools 1-4 before (upper panel) and after CD62L depletion in one representative RNA+ individual (CH9; of four tested) (middle panel= CD62L- PBMC; lower panel= CD62L+ fraction).

For depletion, ELISpot (including non-depletion) was completely repeated in this patient (i.e. raw data of this patient in the table 5.2. are from a different assay previously not including the depletion), Example for correcting for the number of CD4<sup>+</sup> T cells after CD62L depletion: number of IFN- $\gamma$  SFCs/10<sup>6</sup> PBMCs in this patient tested with core P1-4= 135 SFCs/200,000 in CD62L-depleted PBMCs (corrected= 470/10<sup>6</sup> PBMCs compared with 125/10<sup>6</sup> PBMCs in the undepleted sample; see Methods). Values in brackets show range of SFC/10<sup>6</sup> PBMC for all four tested individuals. Depletions were also performed in 3 RNA- individuals, but due to initial technical problems cells were not completely depleted in these individuals. Therefore interpretation of results was difficult to assess.

### ***3.2.5. Correlation between IL-2 and IFN- $\gamma$ secretion***

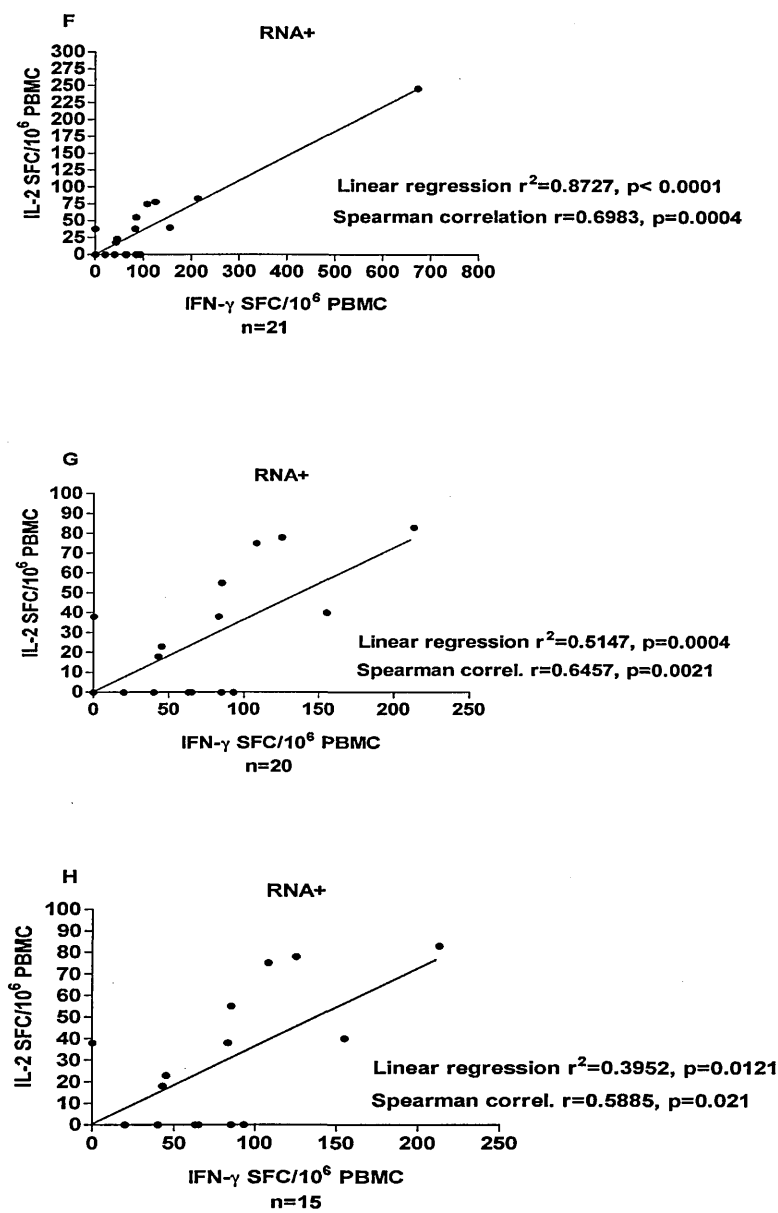
As already done in chapter 4 , here, again I was interested to see whether there was a correlation between IL-2 and IFN- $\gamma$  secretion in these groups. Unlike in chapter 4, where a strong correlation could be observed for the two cytokines in the RNA- group, here, a correlation was not significant in the newly tested RNA- group ( $r= 0.5758$ ,  $p=ns$ , Spearman test; Fig. 5.1.E). Interestingly, in the RNA+ group, this time a strong correlation between IL-2 and IFN- $\gamma$  secretion could be seen ( $r=0.6457$ ,  $p=0.0021$ , Spearman test; Fig. 5.1.F+G). Even when RNA+ individuals with IL-2/IFN- $\gamma$  double-negative values were excluded from analysis, a correlation was still significant in this group ( $r=0.5885$ ,  $p=0.021$ , Spearman test; Fig. 5.1.H).



**Fig. 5.1.E. Correlation between IL-2 and IFN-γ secretion in RNA- group.**

Core pool 1-4 and NS3-5 responses in the IL-2/IFN-γ ELISpot shown for 10 RNA- individuals. For each individual, Core pool 1-4 and NS3-5 responses are summed. One spot represents one individual. Spearman test in parallel with the linear regression as an alternative were performed.

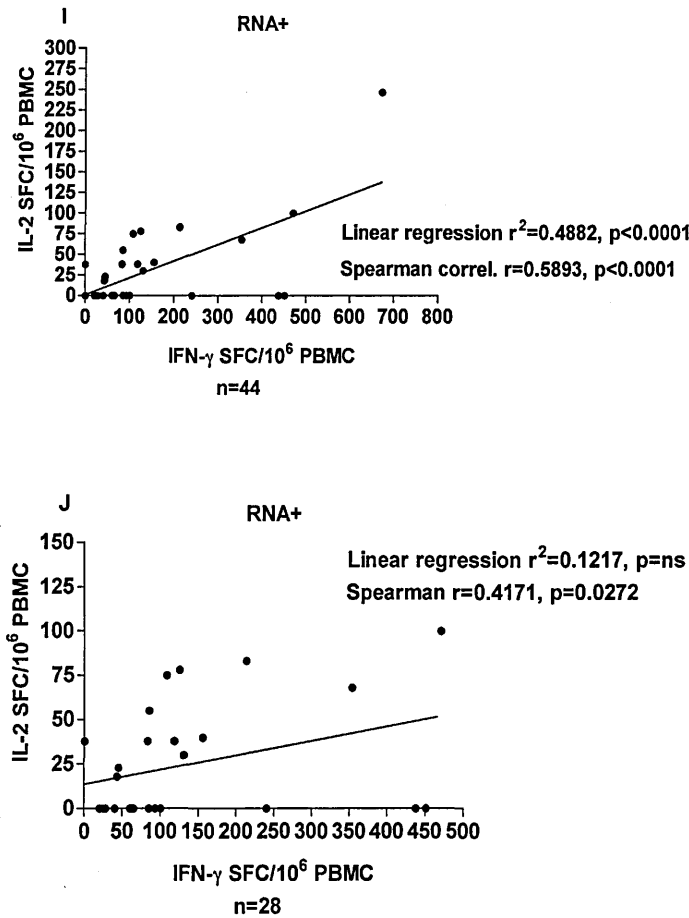




**Fig. 5.1.F+G+H. Correlation between IL-2 and IFN- $\gamma$  secretion in RNA+ group.**

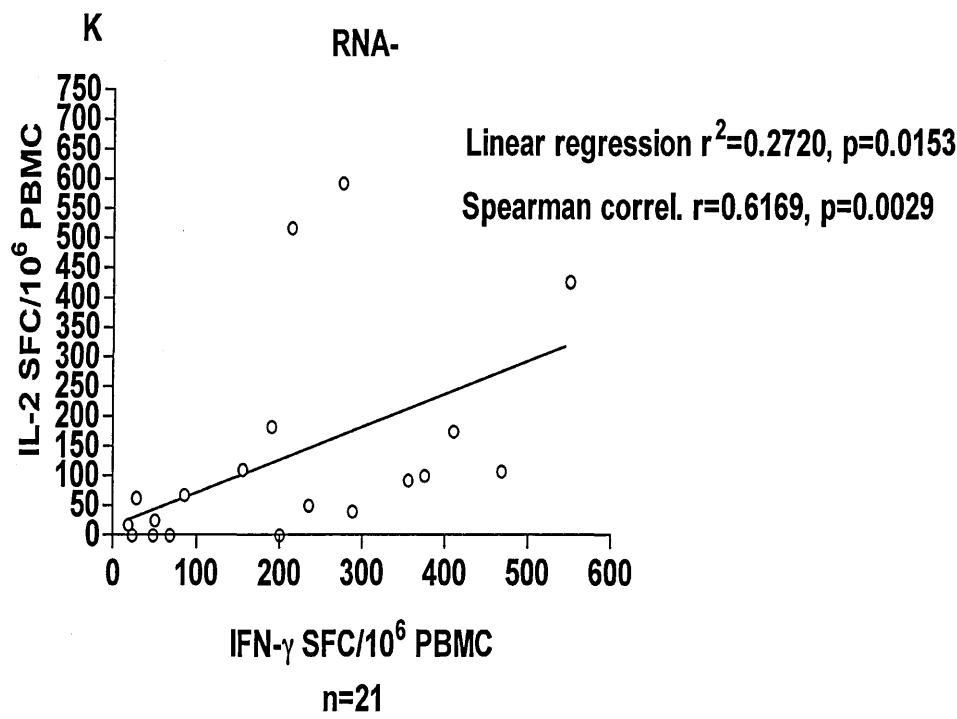
F, Core pool 1-4 and NS3-5 responses in the IL-2/IFN- $\gamma$  ELISpot shown for 21 RNA-individuals (upper panel) including also patients with IL-2/IFN- $\gamma$  double-negative values and one patient with very high IL-2/IFN- $\gamma$  values (240/673 SFC/10<sup>6</sup> PBMC; CH2). G, same as in F, but here the patient CH2 was excluded from analysis (middle panel). H, same as F, but individuals with IL-2/IFN- $\gamma$  double-negative values and patient CH2 were excluded from analysis (lower panel). For each individual, Core pool 1-4 and NS3-5 responses are summed. One spot represents one individual. Spearman test in parallel with the linear regression as an alternative were performed.

When responses from this chapter were analysed with those from chapter 4, a strong correlation between IL-2 and IFN- $\gamma$  secretion was not only seen in the RNA+ individuals ( $r=0.5893$  and  $0.4171$ ,  $p<0.0001$  and  $p=0.0272$ , respectively; Fig. 5.1.I+J), but this time also in the RNA- individuals ( $r=0.6169$ ,  $p=0.0029$ ; Fig. 5.1.K).



**Fig. 5.1.I+J. Correlation between IL-2 and IFN- $\gamma$  secretion in RNA+ groups (chapter 4+5).**

I, IL-2 and IFN- $\gamma$  responses of 23 RNA+ individuals from chapter 4 and of 21 RNA+ individuals from this chapter were analysed together, including also individuals with IL-2/IFN- $\gamma$  double-negative values and patient CH2. J, same as I, but individuals with IL-2/IFN- $\gamma$  double-negative values and patient CH2 were excluded. For each individual, Core pool 1-4 and NS3-5 responses are summed. One spot represents one individual. Spearman test in parallel with the linear regression as an alternative were performed.



**Fig. 5.1.K. Correlation between IL-2 and IFN-γ secretion in RNA- groups (chapter 4+5).**

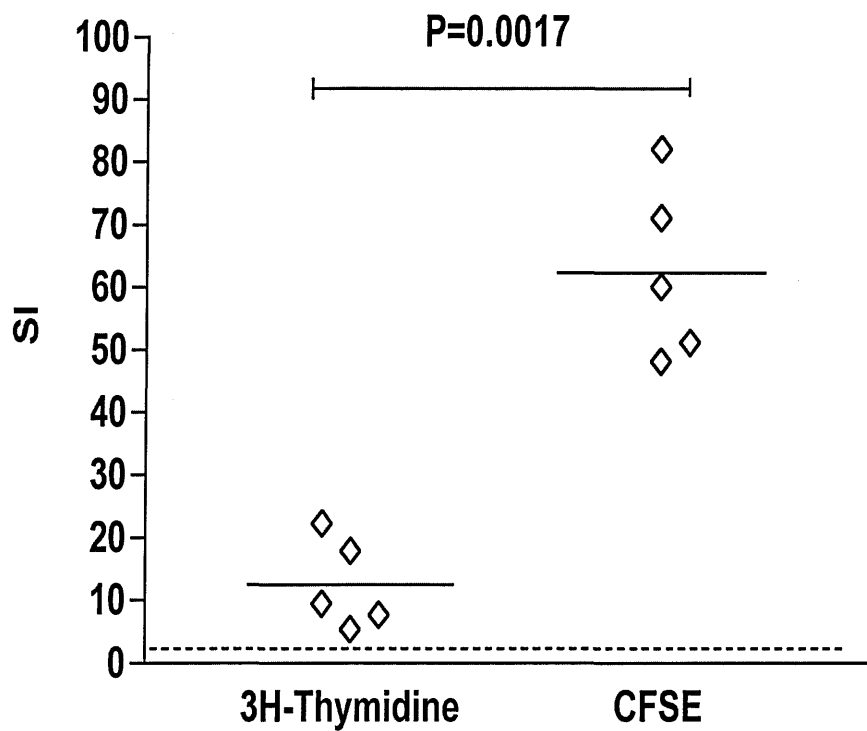
IL-2 and IFN-γ responses of 11 RNA- individuals from chapter 4 and of 10 RNA- individuals from this chapter were analysed together. For each individual, Core pool 1-4 and NS3-5 responses are summed. One spot represents one individual. Spearman test in parallel with the linear regression as an alternative were performed.

Overall, using the pooled groups, and analysing individuals where a response was detectable, we found a correlation in both groups (Fig, 5.1 J+K). However, a linear relationship was only <sup>significant</sup> apparent in the RNA- group.

### 3.3. Comparison of CFSE and $^3\text{H}$ -thymidine assays for detecting T-cell proliferation

#### 3.3.1. *CFSE assay is more sensitive than $^3\text{H}$ -thymidine assay*

The proliferative responsiveness of the  $\text{CD4}^+$  T cells in the two groups was assessed. Initially, the CFSE assay was compared to the more commonly used  $^3\text{H}$ -thymidine incorporation assay. Proliferative responses to cytomegalovirus, a persistent virus associated with expanded pools of effector memory T cells, were analysed. Figure 5.2 shows the proliferative capacity using PBMC from previously CMV-positive tested healthy donors from my lab ( $n=5$ ), cultured with CMV-lysate in a CFSE and a  $^3\text{H}$ -thymidine assay in parallel. Responses were detected for all donors in both assays. However, CFSE yielded significantly stronger responses than the  $^3\text{H}$ -thymidine assay ( $P=0.0017$ ; paired t test), confirming previous studies using tetanus toxoid (Mannering et al., 2003). Results are shown as SI for better direct comparison of the two assays. For the CFSE assay the mean percentage of proliferating CMV-specific  $\text{CD4}^+$  T cells was 54.7% (data not shown).



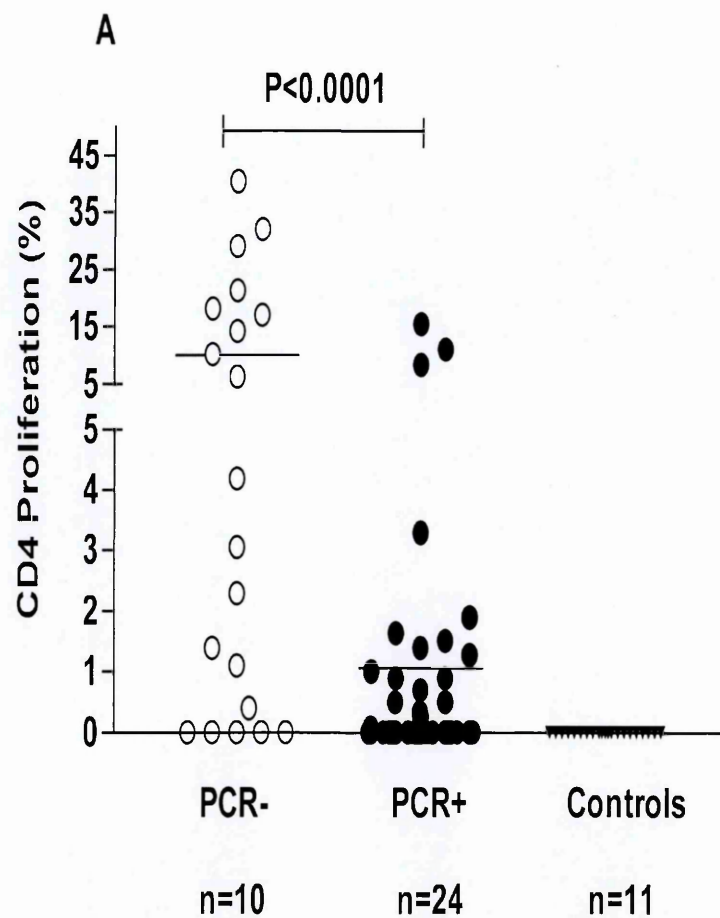
**Fig. 5.2. Comparison of CFSE and <sup>3</sup>H-thymidine incorporation assays.**

CFSE and <sup>3</sup>H-thymidine incorporation assays were set up in parallel with PBMC from five known CMV-responding healthy donors tested using the CMV-Lysate antigen (CMV-L) and stimulated for six days. Responses are shown as stimulation index (SI) as described in the Methods. Horizontal dotted line indicates an SI of 2.

### **3.4. HCV-specific CD4<sup>+</sup> T cell proliferative responses in RNA- and RNA+ individuals**

#### ***3.4.1 Reduced magnitude of proliferative responses in RNA+ group***

To determine the proliferative capacity of HCV-specific CD4<sup>+</sup> T cells at the highest sensitivity at the single-cell level, I stained PBMC with the fluorescent dye CFSE and determined the CFSE content of CD4<sup>+</sup> T cells after 6 days of *in vitro* stimulation with HCV core peptide pools 1-4 or the non-structural proteins NS3-5. Responses were detected in 9/10 RNA- individuals and in 14/24 RNA+ individuals (P value not significant; Fisher's exact test). However, using this assay, CD4<sup>+</sup> proliferative responses in the RNA- group were significantly stronger than in the RNA+ group (10.06% vs. 1.065%;  $P < 0.0001$ ; Mann-Whitney test; Fig. 5.3A).



**Figure 5.3.A. HCV-specific CD4<sup>+</sup> proliferative responses as determined by the CFSE assay.**

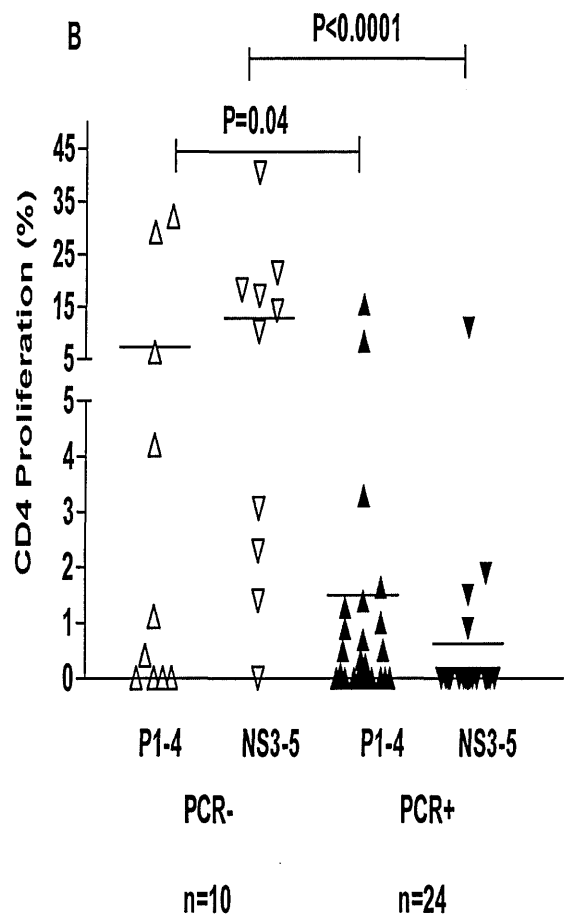
PBMC were stimulated using HCV core peptide pools 1-4 and HCV non-structural proteins NS3/4 and NS5 as described in the methods.

Responses represent the percentage of proliferating CD4<sup>+</sup> T cells after subtraction of the background and responses of core peptides and NS proteins are displayed together.

Responses were compared for 24 HCV+, 10 RNA- individuals and 11 controls. The calculation for the percentage of proliferating CD4+ T cells is described in the Methods. PCR+ = RNA+; PCR- = RNA-

**3.4.2. Lack of proliferation in RNA+ group focused on HCV non-structural proteins**

The differences between the two groups were maintained when we analysed responses for different specificities (Fig. 5.3B). The proliferative responses in the RNA+ group against NS3-5 were rare when compared with the core peptide pools (4/24 vs. 14/24,  $P=0.0065$ ; Fisher exact test). No responses using the CFSE assay were detectable in the healthy control group (Fig. 5.3A).

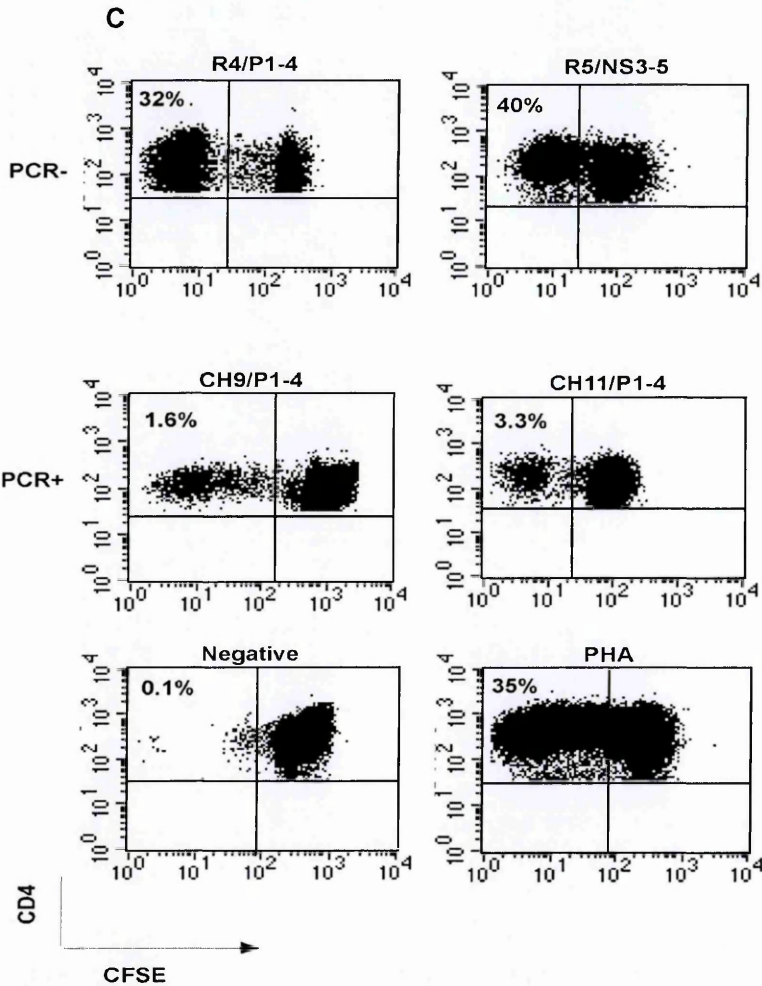


**Fig. 5.3.B. HCV-specific CD4<sup>+</sup> proliferative responses as determined by the CFSE assay.** Responses of core peptides and NS3-5 proteins for each group are displayed separately. Where an SI < 2 was obtained, the frequency of CD4<sup>+</sup> proliferating cells was set as zero, as described in the Methods. PCR+ = RNA+; PCR- = RNA-



3.4.3. Proliferative responses are mainly CD4-derived

PBMC were stained for both CD4 and CD8 in all CFSE assays. Analysis showed the responses were mainly CD4-derived (Fig. 5.3C, CD8 data not shown), confirming previous data using CD8 depletions (Barnes et al., 2002; Semmo et al., 2005b).



**Fig. 5.3.C. Proliferation of HCV-specific CD4<sup>+</sup> T cells.** Shown for 2 RNA- (R4 and R5, upper panel) and 2 RNA+ (CH9 and CH11, middle panel) individuals. CFSE-labelled PBMC on day 6 of following stimulation with HCV core P1-4 and/or NS3-5 proteins are shown. Undivided CD4<sup>+</sup> T cells are detected in the upper right quadrants of each FACS plot and the CFSE signal is diluted with each cell division as the dye is distributed to the daughter cells. Numbers in the upper left quadrants of each plot represent the percentage of HCV peptide- or protein-specific CD4<sup>+</sup> T cells that have proliferated during the 6-day culture.

A negative and one positive (PHA) control are shown in the lower panel. Cells are gated on CD4+ and Viaprobe negative cells. For all tested RNA- individuals median was 3.6% and range was 0-40%. For RNA+ individuals median was 0% and range was 0-15.5% (see also Table 5.2).

3.4.4. Mapping of CD4+ peptides using CFSE

Figure 5.3D shows an example mapping the core peptide pools down to individual peptides in RNA+ individuals using CFSE assay. Responses are shown for one RNA+ individual (CH8) who responded to two peptides of the pools 1-4. Interestingly, this patient was infected with genotype 3. But of the 5 individuals infected with genotype 3, this was the only one with proliferative responses (see Table 5.2).

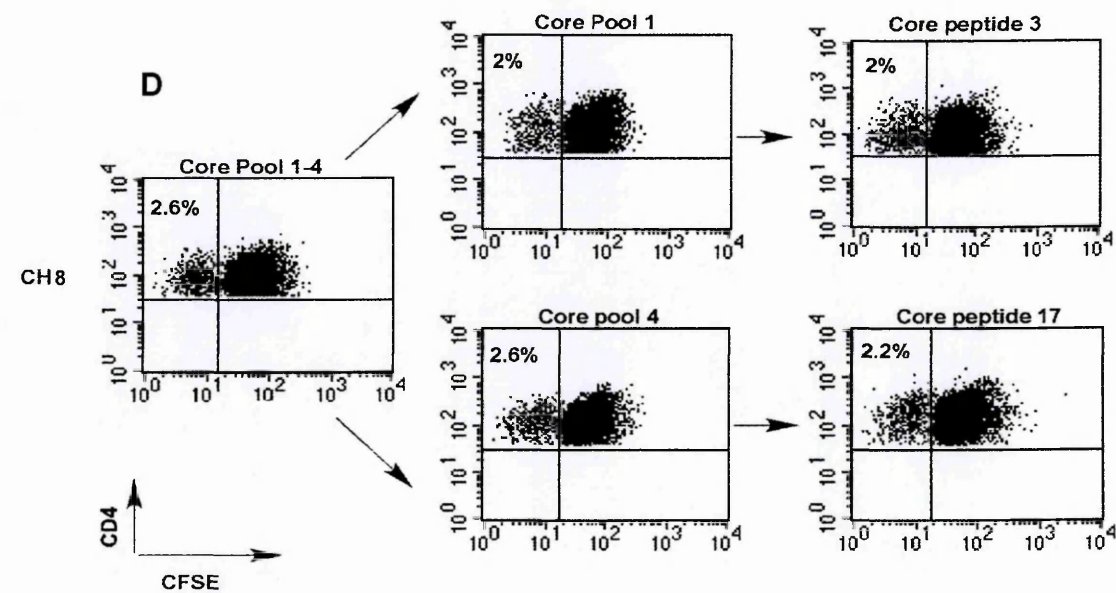
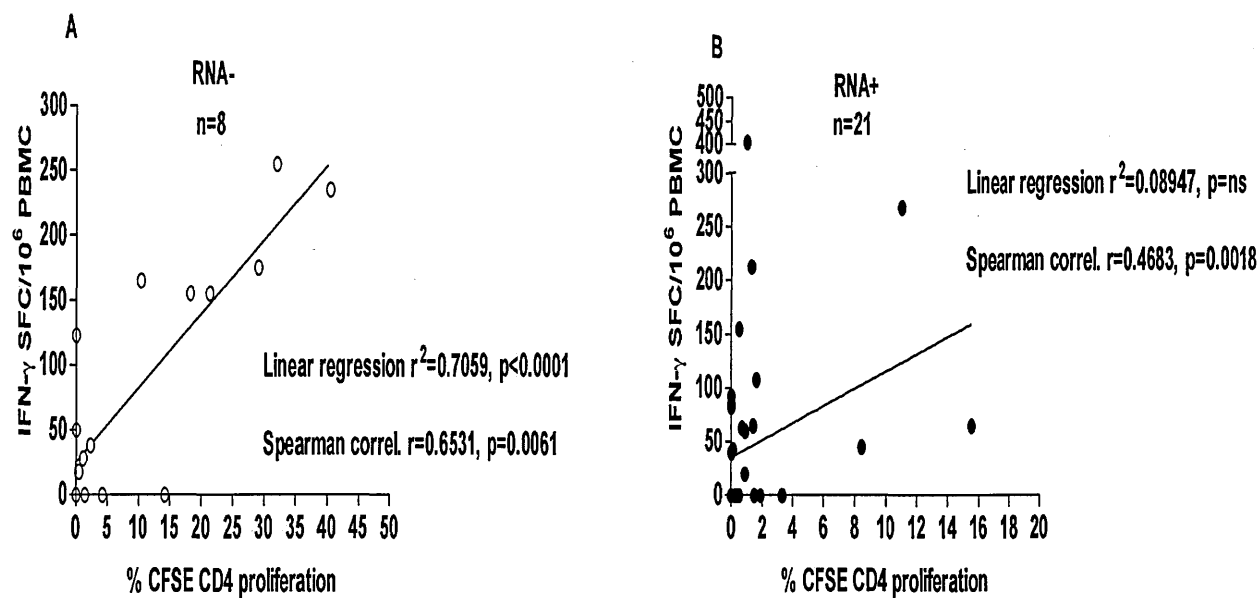


Fig. 5.3.D. Mapping of core peptide pools down to individual peptides in RNA+ individuals using CFSE assay.

Responses are shown for one RNA+ individual (CH8) displaying core peptide pools 1-4 (left hand panel). PBMC were then stimulated with the core pools separately (middle two panels) and eventually all 17 peptides were tested separately as well (right two panels). Responses shown are for core pool 1 and 4 and peptides 3 and 17. Pools 2 and 3 and the other 16 peptides were negative (not shown). For peptide mapping, the CFSE assay in this patient was repeated. Therefore, overall Core pool 1-4 response differs from that in table 5.2.

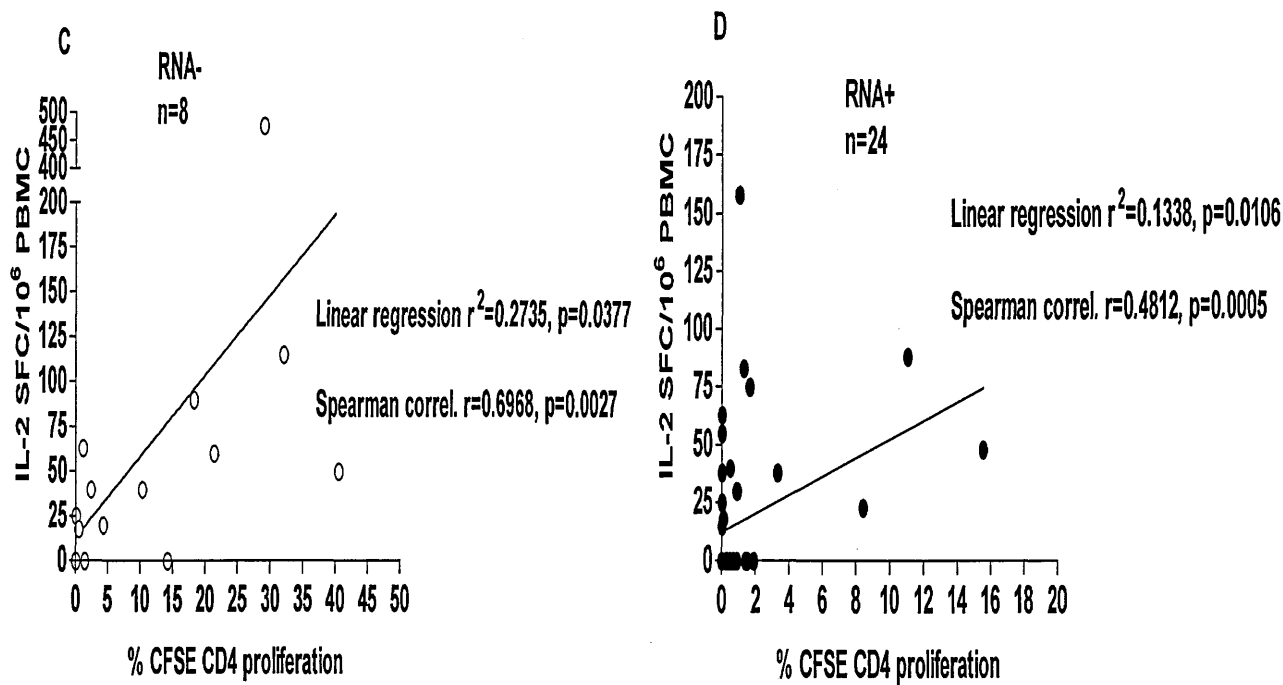
### 3.5. Correlation of cytokine secretion and proliferative capacity

Although much weaker than in the RNA- group, we were able to define proliferative responses in the RNA+ group with the sensitive CFSE assay. We next assessed what relationship existed between the magnitude of such responses and the magnitude of the cytokine responses. Fig. 5.4A demonstrates a very close relationship between the presence of IFN- $\gamma^+$  secreting cells and proliferative responses in the RNA- group ( $P=0.0061$ ,  $r=0.6531$ ,  $n=8$ ; Spearman test), and a weaker but significant association in the RNA+ group (Fig. 5.4B,  $P=0.0018$ ,  $r=0.4683$ ,  $n=21$ ; Spearman test). Additionally, a significant correlation between IL-2-secreting cells and proliferation existed for the RNA- group ( $P=0.0027$ ,  $r=0.6968$ ,  $n=8$  Fig. 5.4C; Spearman test). Interestingly, a positive association existed also for the RNA+ group ( $P=0.0005$ ,  $r=0.4812$ ,  $n=24$  Fig. 5.4D; Spearman test). Thus proliferation was substantially reduced in the RNA+ group, in parallel with the weak IL-2 production in these CD4 $^+$  T cell populations.



**Fig. 5.4.A+B. Correlation between the frequency of HCV-specific CD4<sup>+</sup> T cells monitored by IFN- $\gamma$  in the ELISpot and the frequency of CFSE<sup>low</sup> CD4<sup>+</sup> T cells after 6-day in vitro proliferation.**

Shown for eight resolved (16 dots) and 21 (42 dots) HCV+ individuals tested against the HCV core peptide pools 1-4 and the non-structural proteins NS3-5. 2 dots (one dot for core pools 1-4 and one for NS3-5) represent one individual. For RNA- group only 15 dots are visible as the 16<sup>th</sup> dot goes through zero. For RNA+ individuals, much more dots go through zero (see table 5.2). Spearman test and linear regression as an alternative were performed in parallel.

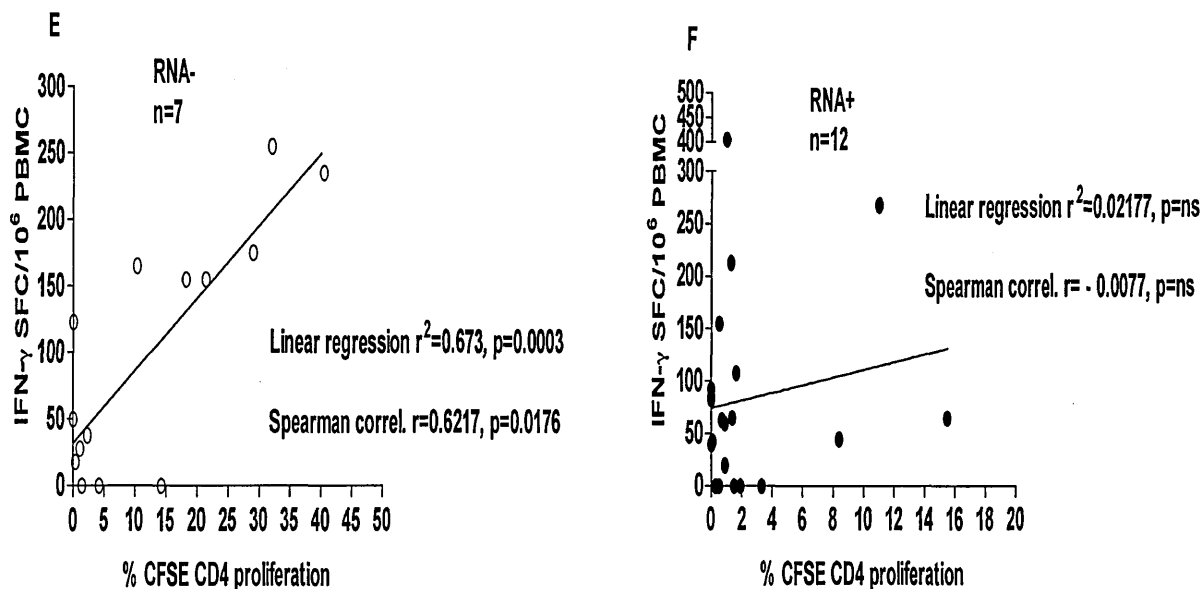


**Fig. 5.4.C+D. Correlation between the frequency of HCV-specific CD4<sup>+</sup> T cells monitored by IL-2 in the ELISpot and the frequency of CFSE<sup>low</sup> CD4<sup>+</sup> T cells after 6-day in vitro proliferation.**

Same correlation as in Fig. 5.4.A+B, but here comparing the proliferation with IL-2 secretion in 8 (16 spots) RNA- and 24 (48 spots) RNA+ individuals. Only 14/16 spots in RNA- group are visible as 2 further spots go through zero. For RNA+ group, 25 spots go through zero. Spearman test and linear regression as an alternative were performed in parallel.

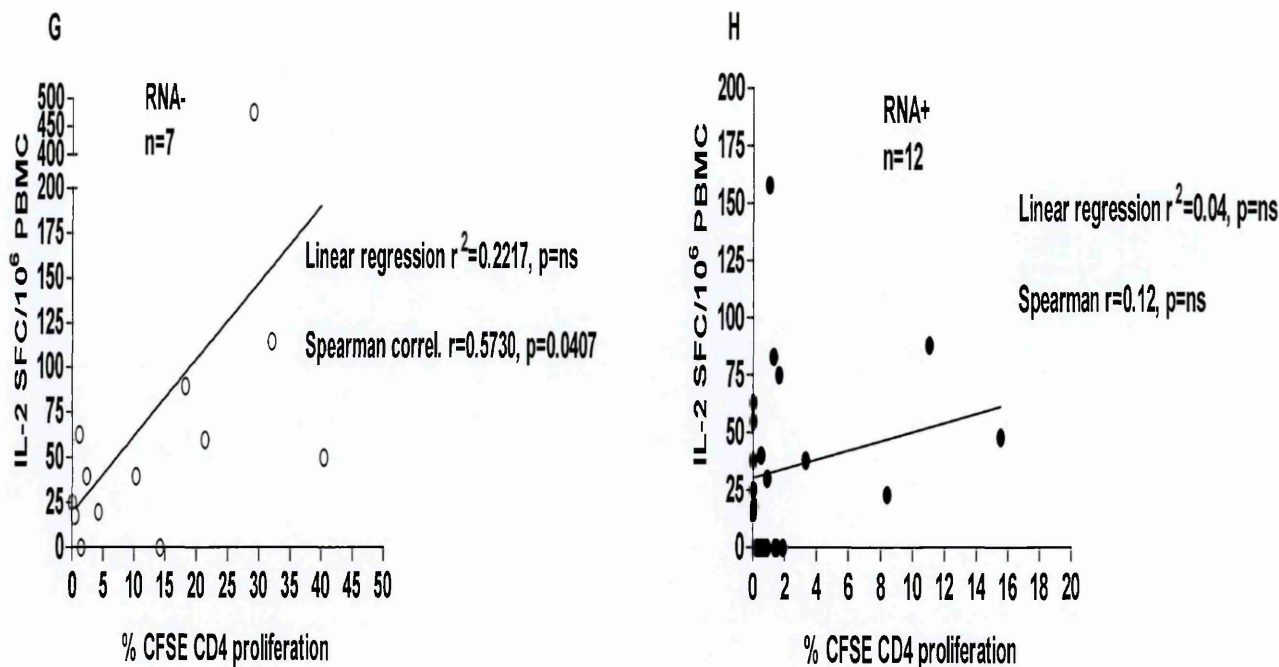
One has to note that especially in the RNA+ group there are many individuals with cytokine/proliferation double-negative values, which might bias the analysis. I therefore was interested to see whether the removal of these “zero” values in both groups might have an influence on the overall analysis.

Fig. 5.4.E confirms the close relationship between the presence of IFN- $\gamma$ <sup>+</sup>-secreting cells and proliferative responses in the RNA- group ( $P=0.0176$ ,  $r=0.6217$ ,  $n=7$ ; Spearman test), but no significant association in the RNA+ group (Fig. 5.4.F,  $P=ns$ ,  $r=-0.0077$ ,  $n=12$ ; Spearman test). A maintained significant correlation between IL-2-secreting cells and proliferation was again observed in the RNA- group ( $P=0.0407$ ,  $r=0.5730$ ;  $n=7$ ; Fig. 5.4.G; Spearman test). The weak, but positive association in the RNA+ group was not observed anymore after the exclusion of “zero” values ( $P=ns$ ,  $r=0.12$ ;  $n=12$  Fig. 5.4.H; Spearman test).



**Fig. 5.4.E+F. Correlation between the frequency of HCV-specific CD4<sup>+</sup> T cells monitored by IFN- $\gamma$  in the ELISpot and the frequency of CFSE<sup>low</sup> CD4<sup>+</sup> T cells after 6-day in vitro proliferation after exclusion of “zero” values.**

Same as for figures 5.4.A-D. But here, individuals with IFN- $\gamma$ /CFSE double-negative values were excluded from analysis. HCV+ individuals tested against the HCV core peptide pools 1-4 and the non-structural proteins NS3-5. 2 dots (one dot for core pools 1-4 and one for NS3-5) represent one individual. 7 RNA- (14 spots) and 12 RNA+ (24 spots) individuals are left for analysis after excluding those with double-negative values. Spearman test and linear regression as an alternative were performed in parallel.



**Fig. 5.4.G+H. Correlation between the frequency of HCV-specific CD4<sup>+</sup> T cells monitored by IL-2 in the ELISpot and the frequency of CFSE<sup>low</sup> CD4<sup>+</sup> T cells after 6-day in vitro proliferation.**

Same as for figures 5.4.A-D. But here, individuals with IL-2/CFSE double-negative values were excluded from analysis. 7 RNA- and 12 RNA+ individuals are left for analysis after excluding those with double-negative values. Spearman test and linear regression as an alternative were performed in parallel.



### 3.6. Precursor frequency, proliferation index and division index

#### ***3.6.1. Loss of proliferation in RNA+ group is due to reduced precursor frequency and not to number of divisions***

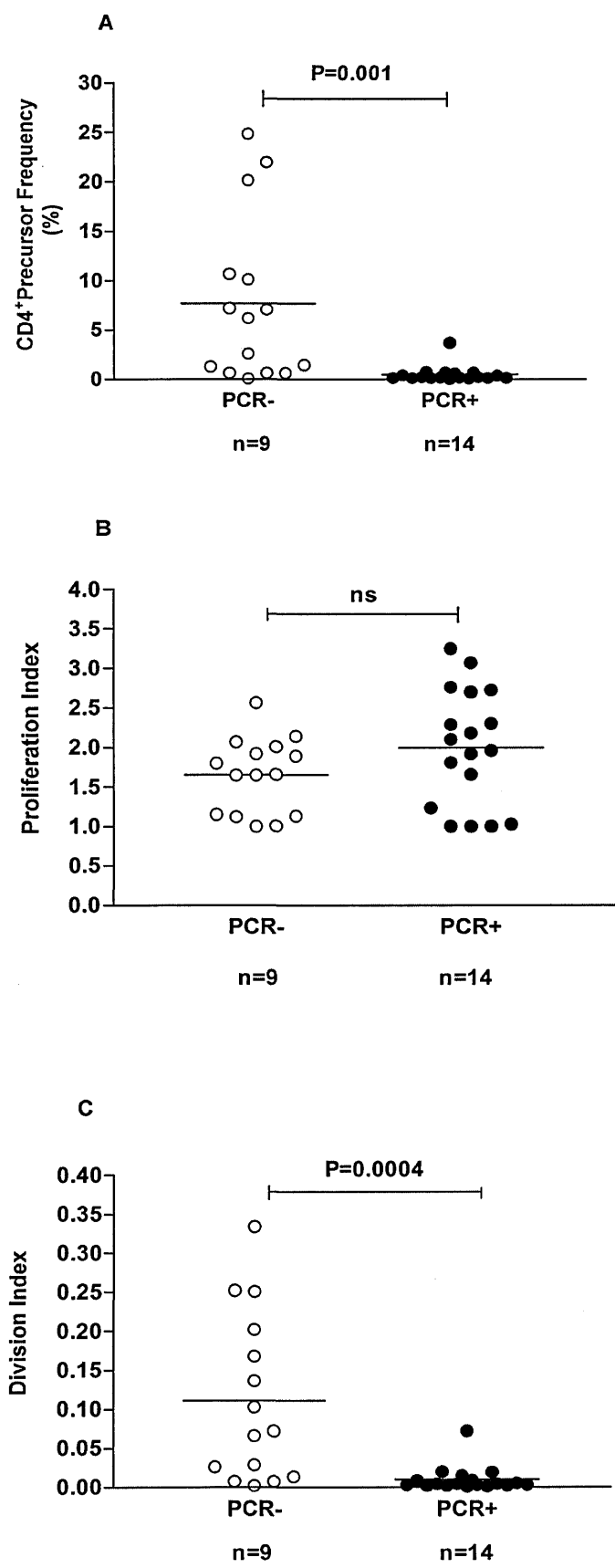
To establish if the loss of proliferation (as % of dividing CD4<sup>+</sup> T cells) was due to a reduction in the number of cells initiating cell division, or due to a reduced number of divisions completed, the profiles of dividing populations were analysed in detail. We assessed the estimated precursor frequency, the proliferation index (average number of cell divisions in dividing population) and the division index (average number of cell divisions amongst all CD4<sup>+</sup> T cells). We found a strongly significant loss of precursors (mean 0.48% vs. mean 7.7%,  $P=0.0002$ ; Mann-Whitney test; Fig. 5.5A) but no difference in proliferation index (mean 1.99 vs. mean 1.65,  $P$  not significant; Mann-Whitney test; Fig. 5.5B) in the RNA+ group when compared to the RNA- group. As expected, the division index (which is derived from both ~~two~~ values) was significantly lower in the RNA+ group (mean 0.01 vs. mean 0.11,  $P=0.0003$ ; Mann-Whitney test; Fig. 5.5C). Thus the defect in cellular proliferation lies at the level of precursors, rather than limitations on the number of divisions undergone.

Interestingly, 3 RNA- individuals had a precursor frequency >20% as shown in Fig. 5.5.A. which, when compared with the other individuals' frequencies, seems to be unusually high. But the significant difference between the two groups was still maintained even after exclusion of these 3 high values ( $P=0.0012$ ; data not shown).

**Fig. 5.5.A+B+C. CFSE Precursor frequency, Proliferaton index and Division index.**

(A) Precursor frequency: The percentage of the cells of the original sample which divided (as calculated according to the methods) is shown for 9 RNA- and 14 RNA+ individuals tested for the HCV core peptide pools 1-4 and the non-structural proteins NS3-5. Precursor frequencies were only included in those with a positive CFSE response (See Fig. 3A and table 5.2). (B) The Proliferation Index is the average number of divisions that those cells, which divided underwent. This is shown again for 9 RNA- and 14 RNA+ individuals tested for the HCV core peptide pools 1-4 and the non-structural proteins NS3-5.

(C) The Division Index is the average number of divisions that a cell that was present in the starting population has undergone. The explanation for 15 spots in 9 RNA- and 18 spots in 14 RNA+ individuals, respectively, is as follows: Of 10 RNA- individuals tested in the CFSE assay, 9 had a response, of whom all had a response to NS3-5 (9 spots). Six of these 9 individuals had also a response to the Core peptide pools 1-4 (6 spots). The patients with responses to both antigen specificities were R1, R2, R3, R4, R6 and R10 (see also Table 5.2). Of the 24 RNA+ individuals tested in the CFSE assay, 14 had a response, of whom all had a response to the Core peptide pools 1-4 (14 spots). 4 of these 14 had also a response to NS3-5 (4 spots). These patients with responses to both antigen specificities were CH2, CH8, CH9 and CH13 (Table 5.2). PCR+ = RNA+; PCR- = RNA-



## 4. Discussion

Understanding failure of CD4<sup>+</sup> T cell activity in HCV is central to understanding viral persistence and thus pathogenesis. There are substantial data to suggest that CD4<sup>+</sup> T cells play a pivotal role in disease outcome: they include immunogenetic studies consistently pointing to a role of MHC class II genes, functional studies in acute human infection, and depletion studies in animal models (Day et al., 2003; Grakoui et al., 2003; Harcourt et al., 2001; Tester et al., 2005; Zavaglia et al., 1998). Furthermore, CD4<sup>+</sup> T cells are emerging as crucial players in other persistent infections such as LCMV, CMV and HIV, although the exact mechanism of their action is not always clear. In HCV, loss of CD4<sup>+</sup> T cell proliferative activity has been recognised for some time as a hallmark of persistent infection (Chang et al., 2001b; Ulsenheimer et al., 2003; Wedemeyer et al., 2002). Studies in acute disease also showed a correlation between loss of CD4<sup>+</sup> T cell responses and re-emergence of virus after transient control, although what is cause and what is effect is controversial (Gerlach et al., 1999; Ulsenheimer et al., 2003). Treatment of chronically infected patients with IFN- $\alpha$ /Ribavirin leads to consistent reconstitution of CD4<sup>+</sup> T cells with proliferative capacity, suggesting that not all T cell responses are deleted but may change functionality (Barnes et al., 2002; Cramp et al., 2000). A single study with MHC class II tetramers specific for selected high avidity CD4<sup>+</sup> T cell responses failed to identify any tetramer-positive cells in persistently infected subjects, indicating that certain responses may be deleted (Day et al., 2003).

As shown in chapter 4 some CD4<sup>+</sup> T cell responses can be detected in chronic infection using *ex vivo* IFN- $\gamma$  ELISpot, although rarely against non-structural proteins (Semmo et al., 2005b).

These core peptide-specific T cells appeared to be low in IL-2 secretion. This raised the issue of whether some of the apparent loss of proliferative responses in chronic HCV may be due to the emergence of T cell populations that are low in proliferative capacity, despite retaining potential to produce IFN- $\gamma$ .

In previous studies using MHC class II-peptide tetramers, it was observed that HCV-specific memory populations in spontaneously resolved individuals are typically CD62L<sup>+</sup> and CCR7<sup>+</sup> (i.e. they have features of “central” memory T cells) (Day et al., 2003). However, in the functional assays performed here I observed that in RNA+ individuals these responses include CD62L<sup>lo</sup> cells. These data are consistent with a recent study of HIV-specific CD4<sup>+</sup> T cells using intracellular staining; the IL-2-low/IFN- $\gamma$ -high cells typical of untreated infections are CCR7<sup>lo</sup> and CD62L<sup>lo</sup> (Younes et al., 2003), i.e. “effector” memory CD4<sup>+</sup> T cells.

Depletion of CD62L<sup>lo</sup> cells probably also removes CD25<sup>+</sup> Treg cells, as CD62L is one of the only known Treg surface markers that is not an activation marker, which is expressed on more than 95% of CD4<sup>+</sup>CD25<sup>bright</sup> cells (Eggena et al., 2005).

Since Treg cells are activated in HCV and since their depletion can influence the responsiveness *in vitro*, as has been shown previously (Boettler et al., 2005; Rushbrook et al., 2005; Sugimoto et al., 2003), it is likely that the very strong response seen after CD62L depletion might in part be influenced by Treg depletion. However, there was consistent IFN- $\gamma^+$  ELISpot reactivity in the CD62L<sup>lo</sup> fraction, and when analysed, the sorted CD62L<sup>high</sup> cells showed little or no reactivity when assayed in parallel (Fig. 5.1D).

The CFSE assay has not been utilised to assess CD4 proliferative capacity in HCV before. This appears to be a sensitive assay *ex vivo*, as previously assessed (Mannering et al., 2003), with the advantage of gating only on CD4<sup>+</sup> T cells, and quantification of a number of features of the dividing populations. It also has the advantages of being simple, robust, independent of HLA type and non-radioactive, and can be readily used in most laboratories. Using this assay, we have observed that some CD4<sup>+</sup> T cell proliferative responses do exist in persistently infected patients, although the frequency of dividing cells is substantially lower than those seen in patients with resolved infection. This proliferative capacity was seen mainly amongst cells specific for core peptides, i.e. the same antigen target as the IFN- $\gamma^+$  responses. Little activity was seen in response to non-structural proteins. However, although antigen-specific proliferation to the same targets was seen, it did not correlate with the magnitude of the IFN- $\gamma^+$  responses in RNA+ patients. This was in stark contrast to the RNA- patients, where a strong correlation was seen.

Interestingly, a correlation was seen for both RNA+ and RNA- patients between antigen-specific IL-2-secreting T cell populations and proliferative capacity. This suggests that the pool of cells retaining proliferative capacity resides within the IL-2<sup>+</sup> population. Alternatively, IL-2 secreted by a subset of antigen-specific cells could contribute to the overall proliferative capacity of the population, regardless of its cytokine status.

An interesting observation in 3 RNA- individuals was a high precursor frequency (>20%) that seemed quite unusually high when compared with that of the other RNA- individuals. Indeed, the estimated precursor frequency in these assays is very high compared to previous estimates for T cell responses using tetramers (Day, 2003). One explanation of this high response could be that T cells responding to peptides do not always respond to the natural antigens. It was (~~Nagvekar et al., 1999~~) found that it is possible, when raising T cell lines against synthetic peptides, to grow cells that do not recognise the naturally processed peptide, possibly because exogenous peptides get loaded unnaturally- eg at wrong pH or without HLA-DM. This could have been the case in some of our patients. Nevertheless, the significant difference in precursor frequency between the two groups was not changed even after exclusion of these three high values. An important issue in general with these estimates is how many of the proliferating cells are actually antigen-specific. Antigen-specific cells, by secreting IL-2 and other cytokines locally, may lead to proliferation of “bystander” cells, which amplifies the response. This issue requires further analysis by study of identified non-HCV-specific (e.g. Flu) cell populations.

Attempts to supplement the CFSE assay with IL-2 led to substantial increases in background (i.e. non antigen-specific proliferation) even with low IL-2 doses (10U/ml). As shown in chapter 4 exogenous IL-2 *in vitro* can restore IFN- $\gamma$  responsiveness in the RNA+ group; it is also possible to reproduce this effect, although much less efficiently, by depletion of CD25<sup>+</sup> populations (which includes T regulatory cells) (Cabrera et al., 2004; Semmo et al., 2005b; Ulsenheimer et al., 2003).

The studies using core peptide-specific responses have been consistent amongst diverse populations and investigators (Barnes et al., 2002; Harcourt et al., 2001; Semmo et al., 2005a; Semmo et al., 2005b). Responses are not seen in HCV-negative controls and are not apparently cross-reactive against non-HCV antigens. The responses to core peptides are not restricted to a single pool or a single peptide. We have mapped these responses down to individual peptides both in previous studies (using IFN- $\gamma$  ELISpot) (Barnes et al., 2002) and here using CFSE (Fig. 5.3D). Although we did not perform sequence analysis here, a prior investigation showed that peptides targetted by HCV-specific CD4<sup>+</sup> T cells were conserved over time and any existing variants in a particular patient were well recognised by endogenous T cells (Barnes et al., 2002). The Core is a conserved gene product and cross-reactivity between different genotypes may occur. It is also possible, as has been observed previously (Harcourt et al., 2003; Kaplan et al., 2005), that largely “historical” responses are detected, due to superinfection with a non cross-reactive strain.



However, the major differences seen between the quality of responses in RNA+ and RNA- groups argue against the RNA+ responses representing only “historical” responses, which have not re-encountered antigen.

The likely explanation for the IFN- $\gamma$ <sup>+</sup>/IL-2<sup>-</sup> /proliferation<sup>lo</sup> status of antigen-specific CD4<sup>+</sup> T cells in the RNA+ group is that these represent a group of cells that continuously re-encounter antigen. This would parallel the experience of those studying HIV infection, and to a certain extent studies of LCMV-specific CD8<sup>+</sup> T cells (Zhou et al., 2004). Interestingly, in a previous study using identical antigens, my colleague Eleanor Barnes et al. noticed a substantial increase in proliferative capacity in patients undergoing IFN- $\alpha$ /Ribavirin therapy, using standard <sup>3</sup>H-thymidine incorporation assays (Barnes et al., 2002). This activity was often unlinked to changes in IFN- $\gamma$  ELISpot, suggesting a relative increase in proliferative capacity, rather than either cytokine secretion or precursor frequency.

Although the interpretation of this is complicated by the actions of IFN- $\alpha$  (antiproliferative, pro-inflammatory) and ribavirin (which modulates DC cytokine secretion) (Barnes et al., 2004a), one simple explanation is that a reduction in viral load leads to a change in functional status of the T cell pools in RNA+ individuals. A future prospective study to analyse proliferation using CFSE and correlate this with IFN- $\gamma$  and IL-2 will be of value in dissecting these striking therapy-related changes, which are consistently observed (Barnes et al., 2002; Cramp et al., 2000; Kamal et al., 2002; Kamal et al., 2004).

Another interesting feature, which has emerged from this and other recent studies, is the dichotomy between core peptide-specific and non-structural protein-specific responses. The assays were originally established to maximise the screening from limited blood volumes, while allowing some investigation of peptide-specific cells. Core was selected as an antigen represented by peptides as this was originally proposed as a significant target of the CD4<sup>+</sup> T cell response (Missale et al., 1997), and available preparations of recombinant core protein have resulted in technical problems regarding background and reproducibility in our hands. Thus one possible explanation for the apparent core-peptide focus of our responses in RNA<sup>+</sup> patients is due to comparing peptides on the one hand and proteins on the other. Despite this, responses in the RNA<sup>-</sup> group typically target both non-structural proteins and core peptides with similar efficacy. An alternative explanation is that these differences reflect distinctive antigen loads associated with a structural vs. a non-structural protein.

Additional studies of E1- and E2-specific responses, using peptide antigens, in comparison to non-structural protein derived peptides (e.g. NS3/4), will help define this further.

Whatever the technical differences, it does appear in RNA<sup>+</sup> patients that some responses are maintained to specific peptides, whereas others become undetectable. Studies using MHC class II peptide tetramers, with magnetic bead enrichment, found no circulating tetramer<sup>+</sup> cells in a small group of HLA DRB1\*0401<sup>+</sup> RNA<sup>+</sup> patients (Day et al., 2003). It is likely that the peptides/tetramers and the enrichment techniques used target high avidity T cells.

It remains possible therefore, that such high avidity T cell responses may be lost, while others, perhaps of lower avidity, are maintained. If so, the quality of the remaining responses as anti-viral effector cells needs further scrutiny. It may be that with high viral loads, the high avidity T cells undergo a process of antigen-induced exhaustion, which “top-slices” the responding CD4<sup>+</sup> T cell pool, leaving only relatively low avidity responses (Ciurea et al., 2001; Oxenius et al., 1998). As has been shown for CD8<sup>+</sup> T cells responding to HIV, it may be that such responses are effectively “passengers”, with little effect on controlling virus (Klennerman et al., 2002b). On the other hand they may play a critical role - further loss of HCV-specific CD4<sup>+</sup> T cell responses in HIV/HCV infection is associated with a substantial rise in viral load (Lauer et al., 2002). Such questions are important in vaccine design, since it might be most important to target high quality/“driver” responses.

Overall, the work in this chapter has provided new insight into an old but crucial problem. Loss of CD4<sup>+</sup> T cell proliferative responses in some cases may be due to exhaustion of CD4<sup>+</sup> T cells and deletion; however, most RNA<sup>+</sup> individuals possess pools of antigen-specific T cells in an IFN- $\gamma$ <sup>+</sup>/IL-2<sup>-</sup> and proliferation<sup>lo</sup> status. To what extent such cells play a role in determining disease outcome, and whether they can be harnessed to prevent disease progression, remain important questions for the future.

## 5. Conclusions

1. **A.** The IFN- $\gamma^+$ /IL-2 $^-$  status in RNA+ individuals confirms data presented in chapter four.  
**B.** Using combined data from chapter four, linear regression and correlation studies indicated overall the strongest association between IFN- $\gamma$  and IL-2 secretion in the RNA- group.
2. This phenotype in the RNA+ group is further characterised by a CD62L<sup>low</sup> status.
3. There is a link between the IL-2 $^-$  status and low proliferation in this cohort.
4. *Ex vivo* demonstration of IFN- $\gamma^+$ /IL-2 $^-$ /CD62L<sup>low</sup>/proliferation<sup>low</sup> is a characteristic of “effector” memory cells.

## CHAPTER 6

# MAINTENANCE OF HCV-SPECIFIC T CELL RESPONSES IN ANTIBODY-DEFICIENT PATIENTS A DECADE AFTER EARLY THERAPY

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## 1. Abstract

**Background:** Early therapy for hepatitis C virus (HCV) is associated with a high rate of viral clearance but the long-term effects on immune responses remain controversial. The role of antibody responses both in initial control of virus and long-term maintenance of immunological memory is not clearly defined.

**Methods:** These issues were investigated in a unique cohort of seven individuals with agammaglobulinaemia who received early interferon therapy after infection through contaminated  $\gamma$ -globulin. T cell responses were measured using a range of assays to study cellular immune function.

**Findings:** A decade later, substantial HCV-specific T cell responses could be recovered.

**Conclusions:** These data indicate that, even in the absence of antibody, early therapy may be associated with long-term maintenance of robust anti-viral cellular immunity.

## 2. Introduction

HCV is a major cause of morbidity and mortality worldwide. Persistent infection is readily established, and is associated with the evolution of liver fibrosis, cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001). The mechanisms behind viral persistence are poorly understood (Rehermann, 2000). The role of antibody in HCV is not clear, as classical in vitro tests of neutralisation are not generally possible. One particular problem for humoral responses is the variability of the target envelope glycoprotein (E2) (Farci et al., 2000). In acute disease progressing on to chronicity, selection for antibody escape mutants within the envelope genes may be observed. Such mutation is not seen in patients with antibody deficiency (Booth et al., 1998). This suggests that antibody may play an important role, in concert with cellular immune responses, but is readily evaded by viral mutation. In the murine model of lymphocytic choriomeningitis virus (LCMV), there are good in vivo data to support a role for antibody in preventing viral persistence (Ciurea et al., 2000). In this infection, cellular immune responses are extremely strong and the neutralising antibody response weak and delayed, yet deficiency in antibody generation ultimately leads to viral recrudescence. The exact role of host-derived antibody in HCV infection is not known – as it is difficult to examine in detail other than in rare antibody- deficient patients.

Common variable immunodeficiency (CVID) is a heterogeneous group of primary antibody production deficiency, characterised by deficient B-cell function (Notarangelo et al., 2004).

A small subgroup of patients has reduced numbers of T-cells, but studies of T cell function show that most patients have good defence against viruses.

In 1994, an outbreak of hepatitis C virus infection, genotype 1a, occurred in 30 hypogammaglobulinaemic patients in the UK from one batch of contaminated anti-HCV screened intravenous immunoglobulin (Chapel et al., 2001; Christie et al., 1997; Healey et al., 1996). Early treatment with interferon- $\alpha$ , 6 million units three times weekly for six months, was initiated within six months of inoculation. The details of the patients and their early treatment with interferon- $\alpha$  have been reported previously (Chapel et al., 2001; Christie et al., 1997; Healey et al., 1996).

I wished to analyse whether immune responses against HCV were maintained after such early therapy, especially in the absence of normal antibody responses. I was able to study seven patients who survived after 10 years, five who remained PCR negative many years after treatment, and two who were still PCR positive.



### **3. Results**

#### **3.1. Study subjects**

Hypogammaglobulinaemic patients were recruited from the John Radcliffe Hospital, Oxford (5 women, 2 men; median age 54 years, range 38-86). The study was conducted according to the ethical guidelines at our hospital and the Helsinki declaration, and approved by the ethics committee at the John Radcliffe Hospital, Oxford. Informed consent in writing was obtained from all participants (five PCR -, two RNA+). Evidence for infection, in particular for those who resolved infection without treatment, were previously elevated liver enzymes and detectable RNA in the acute phase of infection. Details of the individual patients are given in Table 6.1. For detailed methods see methods chapter 2.

Subject	Age	Sex	IFN Treatment	Outcome	ALT (IU/L)
1	53	F	INF- $\alpha$	Resolved	18
2	63	M	INF- $\alpha$	Resolved	16
3	43	F	INF- $\alpha$	Resolved	28
4	38	F	No	Resolved	26
5	52	F	No	Resolved	32
6	86	F	INF- $\alpha$ *	Chronic	89
7	47	M	INF- $\alpha$	Chronic	n.a.

**Table 6.1. Baseline characteristics of the patients**

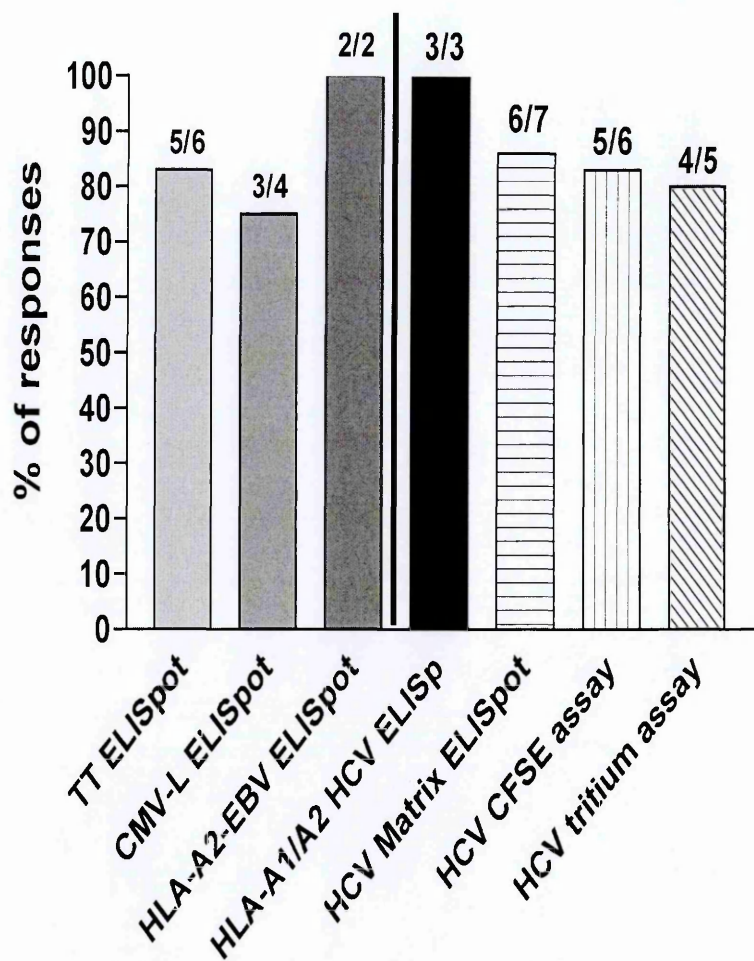
\* Due to intolerance therapy was discontinued early; n.a. = not available  
Viral loads of the two RNA+ individuals were not available.

### **3.2. *Ex-vivo* IFN- $\gamma$ Matrix ELISpot responses to peptides covering the whole HCV sequence**

Table 6.1 shows the baseline characteristics of the patients. Matrix ELISpot analysis of T cells reactive to HCV and control antigens was performed on all hypogammaglobulinaemic patients. HCV Matrix ELISpot responses were demonstrated in 6 of 7 HCV-exposed hypogammaglobulinaemic patients (Fig. 6.1A). Strong HCV-specific responses were detected in all 5 HCV-exposed RNA-negative patients (Fig. 6.1A+B, table 6.2; mean of reactive pools 11, range 4-26 pools; mean magnitude 137 SFC/ $10^6$  PBMC, range 45-360 SFC/ $10^6$  PBMC) and in 1 of the 2 HCV-exposed RNA+ hypogammaglobulinaemic patients (mean 60 SFC/ $10^6$  PBMC; 11 reactive pools).

### **3.3. IFN- $\gamma$ -secreting CD8 $^+$ T cell responses (ELISpot)**

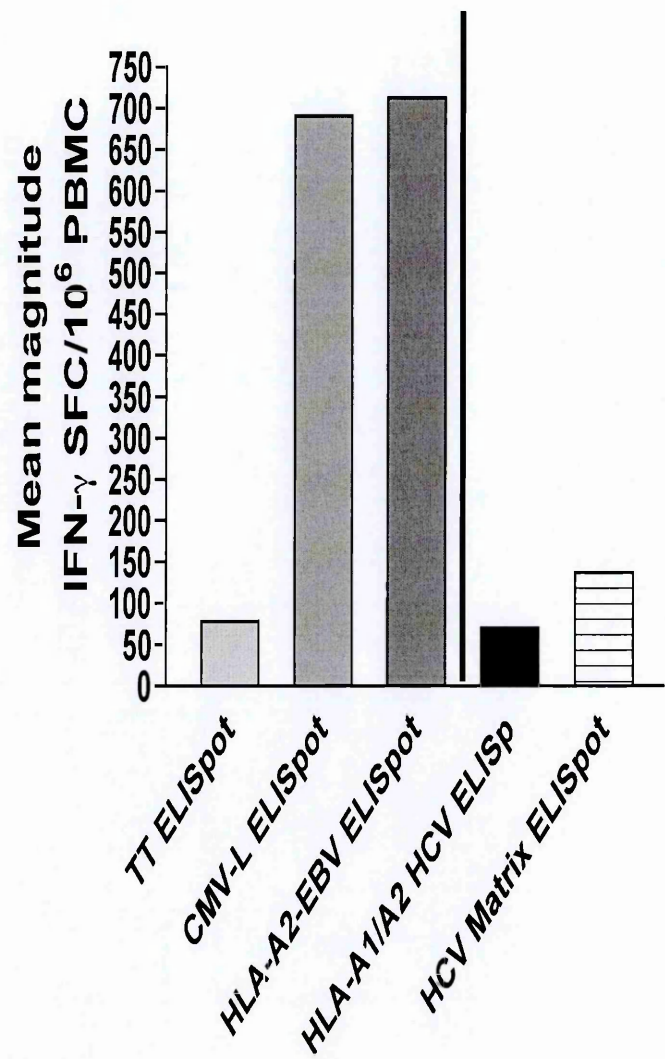
These assays using overlapping peptides could in principle identify both IFN- $\gamma$ -secreting CD4 $^+$  and CD8 $^+$  T cells. To establish if specific CD8 $^+$  T cell responses were sustained these were examined further using HLA class I-restricted peptides in IFN- $\gamma$  ELISpot. These were shown to be CD8 $^+$  T cell memory responses in all three patients tested (2 HLA-A2 and 1 HLA-A1; Fig. 6.1A). These responses are of a magnitude similar to those obtained in previous studies in resolved infection (Lauer et al., 2004) (Fig. 6.1B).



**Fig. 6.1.A. Analysis of HCV-specific and other T cell responses.**

Percentage of positive responses to different antigens tested in a range of techniques in HCV-exposed individuals. Numbers on top of each bar show absolute number of responding individuals tested for the various antigens. Matrix ELISpot = Antigens tested were 58 pools containing 301 overlapping peptides (P1-P58) spanning the whole HCV genome. The numbers include those tested from the whole cohort, both RNA+ and RNA-.

Specific HLA-A2 and HLA-A1 restricted peptides were tested individually according to individual subjects' MHC class I types. These were HCV-NS3 peptide HLA-A2-CINGVCWTV (9mer), EBV HLA-A2-GLCTLVAML (9mer) and HCV HLA-A1-ATDALMTGY (9mer). Subject 1 and 6 had responses to NS3 HLA-A2 and EBV HLA-A2, whereas subject 3 had a response to HCV HLA-A1.



**Fig. 6.1.B. Analysis of HCV-specific and other T cell responses.**

Mean magnitude of antigen-specific T cell responses in ELISpot from same number of patients as in Fig. 6.1.A. Magnitude is shown as IFN-γ SFC/10<sup>6</sup> PBMC, SFC= spot forming cells.

Subject	Matrix ELISpot		ELISpot									
		No. pool responses	TT	SFC/10 <sup>6</sup> PBMC	CMV-L	SFC/10 <sup>6</sup> PBMC	A2-EBV	SFC/10 <sup>6</sup> PBMC	A2-HCV	SFC/10 <sup>6</sup> PBMC	A1-HCV	SFC/10 <sup>6</sup> PBMC
1	+	14/58	+	33	NA		+	708	+	113	NA	
2	+	8/58	-		NA		NA		NA		NA	
3	+	4/58	NA		NA		NA		NA		+	38
4	+	4/58	+	50	+	415	NA		NA		NA	
5	+	26/58	+	168	+	750	NA		NA		NA	
6	+	11/58	+	93	+	910	+	718	+	58	NA	
7	-	0/58	+	125	-		NA		NA		NA	

Subject	CFSE				<sup>3</sup> H Thymidine	
	P1-4	% CD4	NS3-5	%CD4	P1-4	NS3-5
1	+	1.61	+	6.9	-	+
2	+	0.61	+	13.5	NA	NA
3	-	-	-	-	+	-
4	+	1.44	+	0.85	+	+
5	-	-	+	1.35	-	+
6	NA	NA	NA	NA	-	-
7	+	2.3	-	-	NA	NA

**Table 6.2. HCV-specific T cell responses and other tested antigens.**

*Upper table:* IFN- $\gamma$  responses in the ELISpot in 7 hypogammaglobulinaemic individuals tested against different antigens: IFN- $\gamma$  Matrix ELISpot for cytokine secretion testing 58 pools of HCV-specific overlapping peptides; No. pool responses = number of positive pool responses in each individual; TT= tetanus Toxoid, CMV-L= cytomegalovirus lysate, A2-EBV= EBV HLA-A2- GLCTLVAML, A2-HCV= HCV HLA-A2-CINGVCWTV, A1 HCV= HCV HLA-A1-ATDALMTGY; SFC/10<sup>6</sup> PBMC= Spot forming cell per 1 million PBMC; + = positive response, - = no response, NA= not applicable.

*Lower table:* HCV-specific CD4<sup>+</sup> T cell responses using two techniques (CFSE and <sup>3</sup>H thymidine assays for proliferation). % CD4= percentage of HCV-specific proliferating CD4<sup>+</sup> T cells in the CFSE assay.

3.4. HLA Class I tetramer responses

HCV-NS3 peptide HLA-A2-CINGVCWTV (A2-HCV1073) tetramer staining of cells from individual 1 (RNA negative) revealed a clear population of antigen-specific cells that were CD45RO<sup>+</sup>/ CD62L<sup>-</sup>/CD38<sup>-</sup> (Fig. 6.2.A), the same as those previously documented in resolved infection in immunocompetent individuals (Lucas et al., 2004).

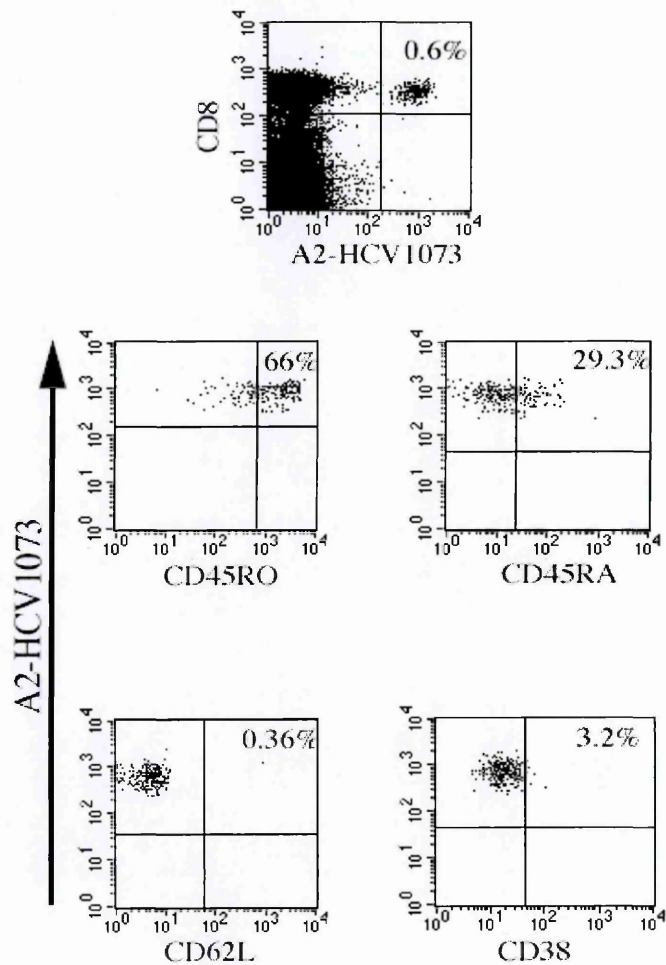


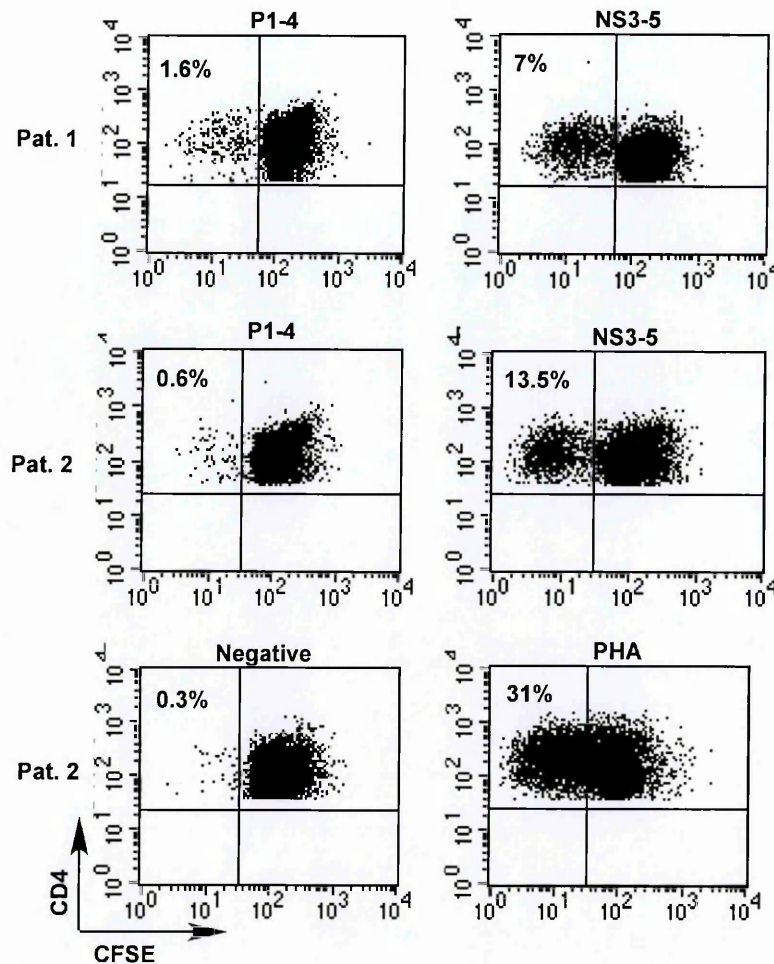
Fig. 6.2.A. Phenotype of cells.

Example of phenotypic analysis on HCV HLA-A2 tetramer positive cells in a HCV-exposed PCR negative hypogammaglobulinaemic individual (Pat. 1) for the markers CD45RO, CD45RA,CD62L and CD38 pertaining to memory status of CD8<sup>+</sup> T cells.

### 3.5. Sustained HCV-specific CD4<sup>+</sup> T cell proliferative responses

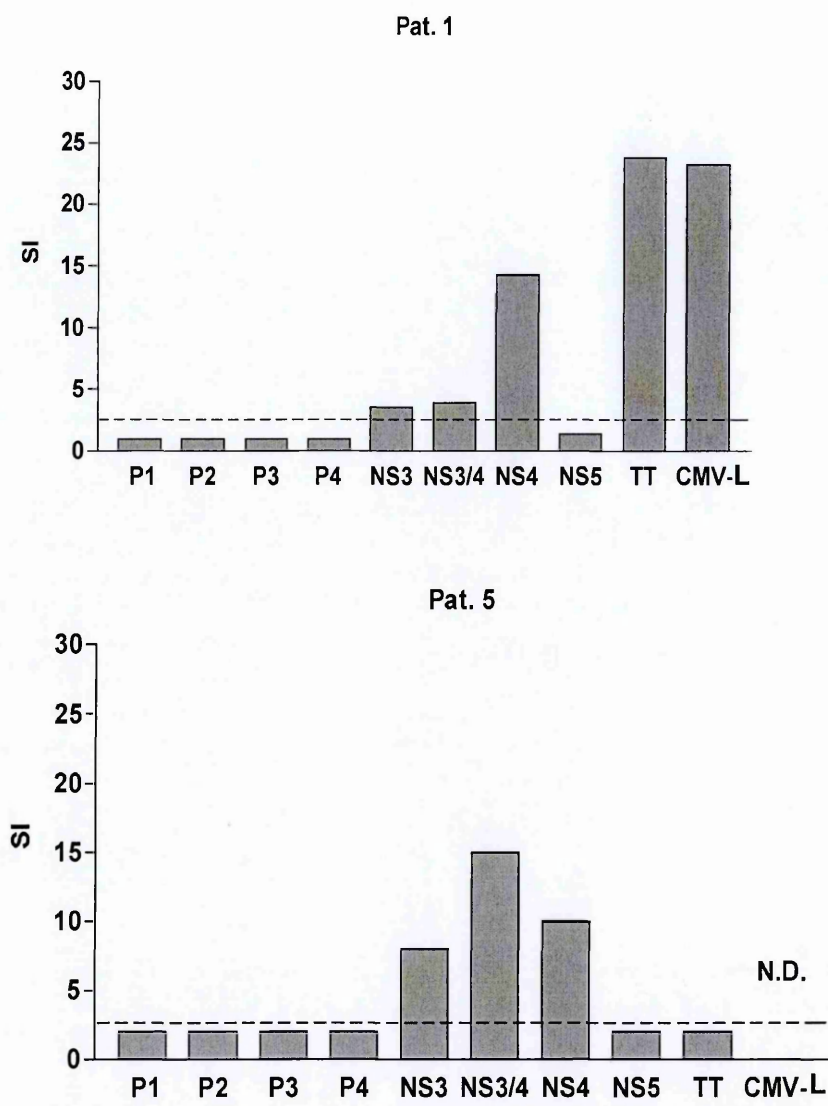
HCV-specific CD4<sup>+</sup> T cell populations were also analysed using a flow cytometric-based assay of cellular proliferation (see methods) (Fig. 6.1A, Fig. 6.2B). This assay confirmed substantial proliferative activity in 5/7 individuals tested. Responses to NS3-5, characteristic of immunocompetent HCV-exposed individuals who resolve infection spontaneously, were observed in 4 of the 5 patients who had resolved the infection and were now HCV-RNA negative. The finding of sustained proliferative capacity, including responses to NS proteins, was also confirmed using conventional <sup>3</sup>H incorporation assays in all patients tested (Fig. 6.2C). Overall, in the group previously exposed to HCV, the 2 RNA<sup>+</sup> patients showed only weak responsiveness in assays of CD4<sup>+</sup> T cell proliferative capacity (1/4 tests positive) compared to RNA<sup>-</sup> patients (12/20 tests positive; table 6.2). Neither RNA<sup>+</sup> individual made a proliferative response to NS proteins, as far as they were tested ,





**Fig. 6.2.B. HCV-specific CD4<sup>+</sup> proliferative responses as determined by the CFSE. Assay.**

Shown for 2 RNA- hypogammaglobulinaemic individuals previously exposed to HCV (Pat. 1 in upper panel, patient 2 in middle and lower panel). PBMC were stimulated for 6 days using HCV core peptide pools 1-4 and HCV non-structural proteins NS3/4 and NS5 as described in the methods. Responses represent the percentage of proliferating CD4<sup>+</sup> T cells after subtraction of the background (Negative). Undivided CD4<sup>+</sup> T cells are detected in the upper right quadrants of each FACS plot and the CFSE signal is diluted with each cell division as the dye is distributed to the daughter cells. Numbers in the upper left quadrants of each plot represent the percentage of HCV peptide- or protein-specific CD4<sup>+</sup> T cells that have proliferated during the 6-day culture. A negative and one positive (PHA) control are shown in the lower panel. Cells are gated on CD4<sup>+</sup> and Viaprobe negative cells.



**Fig. 6.2.C. HCV-specific proliferative responses determined using <sup>3</sup>H thymidine assay.**

Responses shown for 2 RNA- hypogammaglobulinaemic individuals (pat. 1+pat. 5). P1, 2, 3, 4= HCV core peptide pools; TT = tetanus toxoid, CMV-L= CMV-lysate; SI= stimulation index; dashed line represents the SI cut-off of 3 for a positive response. N.D.= not done. CFSE proliferative responses for pat. 1 are shown in Fig. 6.2B. For pat. 5 CFSE CD4<sup>+</sup> T cell proliferative responses were negative for the core peptide pools P1-4 and 1.5% for NS3-5.

Further striking findings are the results for subjects 4 and 5 who spontaneously cleared the virus. Both had IFN- $\gamma$  and proliferative responses (Fig. 6.1.A+B, Table 6.2 and Fig. 6.2C) despite the fact that they neither were treated with IFN- $\alpha$  nor had any antibody responses.

#### **4. Discussion**

In contrast to recently published reports (Rahman et al., 2004; Wiegand et al., 2004), I have shown that a sustained response to anti-viral therapy in acute HCV is associated with a long-lasting HCV-specific T-cell responsiveness in the blood, even ten years after treatment. Such activity was detectable using a variety of methods and included documented CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses.

Two patients in the group did not receive therapy (patients 4 and 5) despite definite exposure, so they controlled the infection spontaneously. These patients had responses in ELISpot and proliferation assays comparable with those in the treated patients. This suggests that HCV-specific T cell responses could be established independently from anti-viral therapy and in the absence of antibodies. In this group it could therefore be speculated that therapy may not be required in all cases.

At the same time, this leads to the intriguing question whether the T cell responses in the treated and resolved individuals (patients 1-3) were therapy-induced or not. At the moment this question is not easy to answer. We therefore have to assume that early treatment in these patients is beneficial.

The responses seen in the interferon- $\alpha$ -treated group are not substantially different from untreated spontaneously resolved hypogammaglobulinaemic individuals, or other spontaneous resolver cohorts with intact B cell repertoires (Lauer et al., 2004; Lechner et al., 2000), although a much larger cohort would be needed to assess this definitively.

These findings shed light on the role of antibody in early therapy and long-term memory. Clearly, early therapy can be effective in the absence of an intact antibody compartment, indicating that its efficacy is not primarily through boosting protective antibody responses. This is in contrast, for example, with therapy of HBV (Rahman et al., 2000). ~~It is, however, consistent with~~ recent data on early therapy for HCV showing success independent of CD8<sup>+</sup> T cell responses (Lauer et al., 2005), *arguing for a direct role for CD4<sup>+</sup> T cell responses*

The role of antibody in long-term T cell memory in man is interesting. In LCMV models, long-term control in the absence of neutralising antibody cannot be sustained (Ciurea et al., 2000; Planz et al., 1997; Thomsen et al., 1996), but there is a prolonged period of control where T cell responses may be maintained at high levels and with a sustained CD62L<sup>low</sup> state (Bachmann et al., 2004). This is thought to be due to low levels of antigen maintaining stimulation of this protective population.

It is possible that very low antigen levels contribute to the maintenance of long-term memory populations in this cohort of patients in the same way, although clearly viral recrudescence does not occur. Single source outbreaks of HCV have been highly informative as to the natural history of HCV infection, and analysis of host-dependent factors (McKiernan and Kelleher, 2000). Here, such an outbreak provided a unique insight into the long-term outcome of early treatment and the longevity of T cell responses in the absence of antibody.

## **5. Conclusions**

1. Early therapy for HCV could be successful even in antibody-deficient patients.
2. Long-term clearance of virus is associated with long-term maintenance of T cell memory in this group.
3. Intact B cell repertoires are not required for long-term maintenance of T cell memory in this cohort.

## **CHAPTER 7**

### **CONCLUSIONS**

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## 1. What was known?

HCV infection is a major cause of liver damage, with virus-induced end-stage disease such as liver cirrhosis and hepatocellular carcinoma resulting in a high rate of morbidity and mortality worldwide. The mechanisms behind viral persistence are poorly understood. Evidence that CD4<sup>+</sup> T cell responses to HCV play an important role in the outcome of infection has been shown in several studies. During chronic HCV infection, HCV-specific CD4<sup>+</sup> T cell responses are weak or absent whereas in resolved infection these responses are vigorous and multispecific. Persons with a T helper type I profile, which promotes cellular effector mechanisms rather than humoral immune responses, are more likely to experience viral clearance.

## 2. What have I learned?

The rate of spontaneous HCV clearance was previously underestimated (20%). Blood donors with RIBA indeterminate serology show evidence of cellular immunity to HCV and therefore prior exposure to antigen. Thus, with these findings one would expect an even higher spontaneous clearance rate. The clearance rate might be even higher than shown in the RIBA indeterminate study (chapter 3). Fifty percent of the blood donors with RIBA indeterminate serology did not show any HCV-specific T cell responses at all. Antigens used were from genotype 1. One possibility could be that this group has cleared virus from a non-genotype 1 infection.

Thus, detection of T cell responses in this group might have been increased with the use of reagents from other HCV genotypes. Alternative more sensitive T cell detection assays, including culture, or enrichment using magnetic beads, might have revealed populations in those who were negative in *ex vivo* ELISpots.

Finally, these findings suggest that RIBA indeterminate blood donors should be counselled appropriately, with respect to potential previous HCV exposure. As in the case of patients with spontaneously cleared HBV infection, I think RIBA indeterminate individuals with cleared HCV infection should be excluded from blood donation, even if the likelihood of infectivity is low. But at the same time, one should give them the reassurance that they are not infectious to anyone else (e.g. their partners).

For a better and quicker diagnosis I think that blood donors with the constellation EIA+/RIBA indeterminate/RNA- should be additionally tested with the ELISpot. This testing is not available in routine clinical practice, but the use of such assays has emerged as clinically very useful in diagnosis of exposure to tuberculosis. Thus in future, the use of ELISpot in clinical testing may become more routine.

The other important finding from these studies is that HCV-specific CD4<sup>+</sup> T cell populations do exist in persistent infection but with reduced proliferative capacity. This status is associated with the production of IFN- $\gamma$  upon antigen stimulation, but little or no IL-2. These cells are CD62L<sup>lo</sup>, suggesting an effector memory phenotype (IFN- $\gamma$ <sup>+</sup>/IL-2<sup>-</sup>/proliferation<sup>lo</sup>/CD62L<sup>lo</sup>).



Failure of IL-2 secretion appears to be an important determinant of the status of CD4<sup>+</sup> T cell populations in chronic HCV infection and may lead to disruption of IFN- $\gamma$  production and proliferative function *in vivo* – a status that characterises the cellular immune response in both CD4<sup>+</sup> and CD8<sup>+</sup> T cell compartments in chronic disease.

Here, I have shown that *in-vitro* addition of IL-2 may help to restore IFN- $\gamma$  secretion in some individuals. Similar results have been described recently in the case of acute HCV infection where the breadth and vigour of the T cell responses were analysed in an Italian cohort with acute HCV (Folgori et al., 2006). Here, as has been shown previously, strong and sustained CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in those with acute resolving infection were observed. In those where immune control was lost, although T cell responses were induced initially, these declined quite rapidly, and became undetectable in the majority of patients. Impairment of proliferation was also a marked feature of such responses in the persistently infected group. However, these responses could be restored *in vitro* by the addition of IL-2. Collapse of proliferative responses could contribute to the failure to resolve infection, since in those with resolving infection, proliferative responses were maintained over time. This again confirms data shown in my studies.

Future clinical studies will have to show whether the addition of IL-2 on top of the commonly used therapy with interferon and ribavirin will also be able to restore IFN- $\gamma$  secretion *in vivo*. Recently, a pilot study treating chronically HCV-infected patients with interferon- $\alpha$ /ribavirin and IL-2 did not exert a favourable impact on HCV treatment.

However, this study included only patients with severe liver disease who previously did not respond to commonly used therapy with interferon- $\alpha$ /ribavirin alone (Alric et al., 2006).

In my studies, responses in persistent infection were primarily detected against core-derived peptides. Unlike in spontaneous resolution, responses against non-structural proteins in persistent infection appeared weaker. Although responses against core peptides and non-structural proteins were detectable to a similar extent in the RNA- group, this constellation, when compared with that in the RNA+ group, suggests that the presence of non-structural protein responses predicts viral clearance. But this suggestion would contradict the findings in the RIBA indeterminate study where responses to core peptides (pool 2) were the leading responses and probably led to clearance of the virus. A weakness of my studies might be the comparison of core peptides with non-structural proteins. A study with peptides for both structural and non-structural proteins would shed more light into this aspect.

Sequences of the core gene and some of the non-structural protein genes, such as NS3, are more conserved than other regions of the HCV genome. If responses to core and/or non-structural proteins predict viral clearance, then these conserved regions might be suitable for the development of T cell vaccines.

The role of antibody responses both in initial control of HCV and long-term maintenance of immunological memory was not clearly defined so far. Here, for the first time, I showed that antibody responses are not necessary to clear the virus. As shown in a group of antibody-deficient patients who were previously infected with HCV, the role of T cell responses in control of virus becomes even more evident.

Two agammaglobulinaemic patients cleared the virus spontaneously without antiviral therapy. This suggests that HCV-specific T cell responses could be established independently from anti-viral therapy and in the absence of antibodies. This suggests that therapy might not be necessary. However, a larger cohort of similar patients is needed to confirm this speculation.

### 3. Model of successful and unsuccessful immunity against HCV

How do these new data help to refine a model of successful and unsuccessful cellular immunity against HCV? Currently the mechanisms underlying the failure to sustain peripheral T cell responses in chronic infection can be considered as 4 main groups:

1. **Escape.** Mutation in T cell epitopes was clearly demonstrated in chimpanzee models (Erickson et al., 2001), although it is only recently that evidence has been really convincing in man. Mutations leading to reduced recognition by CD8<sup>+</sup> T cells has been observed after acute transmission events, in a cohort of acutely infected individuals studied prospectively and in single source outbreaks (Cox et al., 2005; Timm et al., 2004; Ray et al., 2005). It is very likely that most of such escape occurs very early, as judged by data in the animal model. In man, nevertheless, escape does not occur in all CD8<sup>+</sup> T cell epitopes (Cox et al., 2005; Urbani et al., 2005), and responses to all epitopes undergo substantial decline in chronic disease, so alternative mechanisms for T cell downregulation must play some role. The importance of escape in evasion of HCV-specific CD4<sup>+</sup> T cell responses is not currently known.

A MHC class II tetramer study by my colleague Michaela Lucas showed no role for escape in CD4<sup>+</sup> T cells (manuscript submitted). Therefore, regarding CD4<sup>+</sup> T cells in HCV, at the moment escape might play only a minor role in unsuccessful immunity in persistent HCV infection.

2. **Exhaustion.** This term is derived from work originally performed in the murine model of lymphocytic choriomeningitis virus (LCMV) (Moskophidis et al., 1993). In this model, certain viral strains when given in high doses set up high level persistence with long term viremia. This is associated with decline and loss of CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses. The early experiments in this field showed deletion of CD8<sup>+</sup> T cells, but subsequently different investigators have proposed a sliding scale of “partial” exhaustion with progressive loss of IL-2 secretion, proliferation, IFN- $\gamma$  secretion and cytotoxicity occurring, prior to full deletion (Wherry et al., 2004). The mechanism is not fully understood, but it is an antigen-specific effect and probably results from excessive T cell receptor (TCR) triggering, perhaps in the absence of sufficient co-stimulation or help (see below). In HCV, the fact that CD4<sup>+</sup> (as I have shown in my studies) and CD8<sup>+</sup> T cell responses (Lauer et al., 2002) may be rescued *in vitro* with IL-2, suggests that full exhaustion does not occur, but in many respects this model does describe the evolution of T cell responses, since reductions in diverse aspects of function have been reported (Wedemeyer et al., 2002).
3. **Helplessness.** Failure to provide adequate CD4<sup>+</sup> T cell help in murine models also leads to long term failure to control viral infections such as LCMV (Zajac et al., 1999). This “helpless” state is associated with the development of CD8<sup>+</sup> T cell responses, which lack full effector and proliferative function.

Clearly, I have shown failure of CD4<sup>+</sup> T cells in my studies lacking the capacity to secrete IL-2, a crucial helper cytokine. The lack of this helper cytokine was associated with reduced IFN- $\gamma$  production and low proliferation in persistent HCV infection. Thus failure to maintain IL-2 secretion may be a critical factor in the development of a “helpless” state.

However, many CD4<sup>+</sup> T cell responses do appear to be deleted (Diepolder et al., 1995; Day, 2003), and in some cases this is an early event. So if failure of CD4<sup>+</sup> T cell responses is ultimately responsible for failure of CD8<sup>+</sup> T cell responses, this still begs the question as to why HCV-specific CD4<sup>+</sup> T cell responses appear to fail so early.

4. **Regulation.** The above models derive largely from work in the murine model of LCMV, where escape and exhaustion dominate. In other murine models of virus persistence, such as Friend leukaemia virus (FLV), other mechanisms appear important (Dittmer et al., 2004). CD25<sup>+</sup> and/or Foxp3<sup>+</sup> CD4<sup>+</sup> regulatory T cells (Tregs) were originally observed in inflammatory conditions, but clearly play a role in a variety of infections. Tregs may be “natural”, or induced after prolonged antigenic stimulation. It is very likely they play an important role in preventing immune-mediated pathology during chronic HCV. Indeed, studies of blood and liver from a variety of groups have demonstrated regulatory activity, which can influence the proliferation of CD8<sup>+</sup> and CD4<sup>+</sup> T cells *in vitro* (Sugimoto et al., 2003; Cabrera et al., 2004; Boettler et al., 2005; Rushbrook et al., 2005).

The major issue in understanding the role of Tregs in this disease is that by and large, the defect in immune responsiveness is HCV-specific, whereas other peripheral immune responses are left intact. One possible explanation is that, although demonstrable in blood, Treg activity is likely to be concentrated at the site of disease – i.e the inflamed liver.

Further studies of this issue are required to establish to what extent local Treg activity protects against immunopathology and whether it contributes to the disappearance of HCV-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells observed in this most cohorts. Studies in the laboratory by my colleague Scott Ward (manuscript submitted) suggest that approximately 1 in every 2-3 CD4<sup>+</sup> T cells in liver infiltrates is Foxp3<sup>+</sup>.

Overall, the data in my studies add weight to the concepts of exhaustion and helplessness as important factors in HCV-specific T cell failure. Sustained exposure of T cells to viral antigen drives them initially into an “effector” state where anti-viral cytokines such as IFN- $\gamma$  are secreted, but IL-2 secretion is lost. Failure to secrete IL-2 may then contribute to a vicious circle where HCV-specific CD8<sup>+</sup> and CD4<sup>+</sup> responses are induced but proliferation cannot be maintained, so allowing production of more virus – which further enhances the exhaustion.

Escape of virus from key (CD8<sup>+</sup> T cell) immune responses by mutation at an early stage will serve to maintain high levels of antigen and accelerate this process. Ultimately, unless virus is cleared rapidly, cells progress along a downward slope to full exhaustion.

Importantly, however, although all these processes may contribute to the decline of antigen-specific T cell responses, it is still rather difficult to disentangle what is cause and what is consequence of virus persistence.

#### **4. The “liver effect”**

One further element must be considered which I did not address in my studies, but which is nevertheless probably critical. The liver has an important role as an immune regulator, containing specialised antigen presenting cells and populations of non-classical T cells and natural killer (NK) cells with potential antiviral and regulatory functions. However, the size of these populations, and subsets, differ greatly. Natural killer cells constitute the largest group of lymphocytes within the liver. Normally comprising just 2% of the peripheral lymphocytes, NK cells make up 31% of the liver lymphocyte population. NKT-cells are also present in increased numbers; while both T-cells and B cells have a lower presence than seen in the periphery. The CD4:CD8 T-cell ratio is also reversed in the liver, with CD8<sup>+</sup> T-cells comprising just under 63% of the liver T-cell population.

The liver is well known for its capacity for promoting tolerance. It is the only organ that can be transplanted across the MHC barrier by inducing tolerance. Also, a liver transplant somehow ‘protects’ a co-transplanted kidney. This ability to suppress T-cell response has been termed “the liver effect” (Calne R., 2002). Tolerogenicity in the liver may also be a major basis for oral tolerance.

The liver has a number of resident cell types capable of presenting antigen to T lymphocytes, all of which have been implicated in inducing tolerance.



These include the professional antigen-presenting dendritic cells, as well as Kupffer cells, liver sinusoidal epithelial cells (LSECs), and hepatocytes. The expression of MHC Class II allows the presentation of antigen to CD4<sup>+</sup> T-cells and their subsequent activation. However, in some experimental settings some of these possess a T<sub>reg</sub> cell phenotype and express IL-4 and IL-10 (Knolle et al., 1999).

Unlike APCs, presentation of antigen to either naïve CD4<sup>+</sup> or CD8<sup>+</sup> T-cells by LSECs has been shown not to induce differentiation to Th<sub>1</sub> or CTL respectively, but instead induced CD4<sup>+</sup> to express a Th<sub>0</sub> phenotype and CD8<sup>+</sup> dysfunction (Knolle et al., 1999; Limmer et al., 2000). Thus, antigen presentation by LSECs to naïve T-cells induces a tolerogenic effect. This ability to induce tolerance in both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells has recently been shown to cross MHC barriers (Onoe et al., 2005). However, the exact mechanism/s behind this ability remains unclear. On the other hand, a recently published paper has shown that the liver may be an excellent priming site for naïve CD8<sup>+</sup> T cells (Klein et Crispe, 2006). This hypothesis was tested in an optimised orthotopic liver transplantation model. They concluded that local antigen presentation cannot explain liver tolerance. These controversial data make a further and detailed study of the immunological functions of the liver necessary.

## 5. Other limitations of the studies

HCV-specific CD4<sup>+</sup> T cell responses were analysed only from PBMC's. But T cell responses in the liver play also a major role in the outcome of HCV infection. Therefore, many responses in PBMC might have been missed in my studies, probably because they were located in the liver –despite the above described tolerogenic effect of the liver. A previous study showed that intrahepatic HCV-specific CD8<sup>+</sup> T cells are present in the majority of tested patients with chronic HCV infection and overlap significantly with the response present in the peripheral blood (Spangenberg et al., 2005). A large fraction of intrahepatic HCV-specific CD8<sup>+</sup> T cells were impaired in their ability to secrete IFN- $\gamma$ . This dysfunction was specific for HCV-specific CD8<sup>+</sup> T cells, since intrahepatic Flu-specific CD8<sup>+</sup> T cells readily secreted this cytokine.

It is therefore important to study also the frequency and the anti-viral function (IFN- $\gamma$ , IL-2, IL-10 and perforin secretion) of HCV-specific CD4<sup>+</sup> T cell responses in liver and PBMC and compare the quality and quantity of these responses in the two compartments.

Compared to the number of CTL epitopes described, only few CD4<sup>+</sup> T helper epitopes have been identified so far. Identifying new HCV-specific CD4<sup>+</sup> T cell epitopes will not only allow one to address relative T cell frequencies, their role in memory, and epitope-specific cytokine production, but also will help in the development of T cell based vaccine studies.

I have shown that the *in vitro* addition of IL-2 can restore IFN $\gamma$  production in some RNA+ individuals. It would be interesting to see whether or not other cytokines have similar functions. Interleukin 15 (IL-15) is critical for the development of human and murine natural killer (NK) cells and hepatic-derived NK T cells (NKT) in mice, and for the homeostatic maintenance of NK/NKT and CD8<sup>+</sup> memory T cells (Golden-Maisen et al., 2004). In HCV, the reduced effector functions of NK cells were found to be associated with a significant reduction in serum levels of the innate cytokine IL-15. In vitro stimulation with IL-15 rescued NK cells of HCV+ patients from apoptosis and enhanced proliferation and functional activity (Meier UC et al., 2005). Unfortunately, when I tried to restore ELISpot IFN- $\gamma$  production in some of the RNA+ individuals, this caused a high background, probably due to activation of NK cells.

Use of some other techniques might help in future projects to further understand the role of IL-15 in the context of HCV infection.

Other cytokines have been shown to play a role in HCV infection as well. In a murine study, the administration of IL-23 and IL-27 as potent adjuvants resulted in great increases in the number of IFN- $\gamma$ -producing, HCV-specific CD8<sup>+</sup> cells (Matsui et al., 2004). The two novel cytokines might offer new therapeutic strategies against infectious pathogens such as HCV. But the question would be whether they also have a similar effect on HCV-specific CD4<sup>+</sup> T cells.

## 6. Goals of future work

In the last two years, using overlapping peptides, I have been able to identify HCV-specific CD4<sup>+</sup> T cells in persistent infection that recognise the mainly conserved core region. These cells characteristically produce IFN- $\gamma$  but not IL-2 and have a limited ability to proliferate (IFN- $\gamma$ <sup>+</sup>/IL-2<sup>-</sup>/proliferation<sup>lo</sup>). To what extent such cells play a role in determining disease outcome and whether they can be modulated to prevent disease progression remain important questions, which I will attempt to address in future projects.

The aim of future projects should be to further assess the role of virus-specific CD4<sup>+</sup> T cell responses in individuals with HCV infection with and without anti-viral therapy. HCV-specific CD4<sup>+</sup> T cells need to be identified in these groups of patients using overlapping peptides spanning the whole HCV polyprotein and their functional and phenotypic status assessed using a range of techniques. Ideally also, HLA-mapping of key peptides will allow the further dissection of such responses using class II tetramers.

### 6.1. Influence of HCV-specific CD4<sup>+</sup> T helper cells on HCV-specific CD8<sup>+</sup> T cell responses

Effective anti-viral CTL responses in chronic viral infections are critically dependent on virus-specific CD4<sup>+</sup> T helper cells (Boettler et al., 2005; Rushbrook et al., 2005). It will be particularly important in any future studies to see to what extent the CD4<sup>+</sup> T cell function influences the CD8<sup>+</sup> T cell function and in more detail,

whether a dysfunctional CD4<sup>+</sup> T cell response in persistent HCV infection correlates with a dysfunction of CD8<sup>+</sup> T cell responses. Studies investigating the comparison between the two subsets have not been performed in detail. Furthermore, it will be important to show whether there is a correlation between the CD4<sup>+</sup> and CD8<sup>+</sup> responses and the viral load.

## **7. Other studies performed and to be followed up**

### **7.1. Reduced HCV-specific CD4<sup>+</sup> T cell responses in HIV/acute HCV co-infected individuals**

HIV/HCV co-infection is a common clinical problem with high HCV viral load and accelerated progression of liver disease. Up to 80% of HIV+ intravenous drug users and 98% of HIV+ haemophiliac cohorts are co-infected with HCV (Mohsen et al., 2002). Increasing evidence shows that co-infection can affect the clinical course of either disease (Martin-Carbonero et al., 2004). These important clinical effects may arise from virological interactions, or an underlying immunological mechanism.

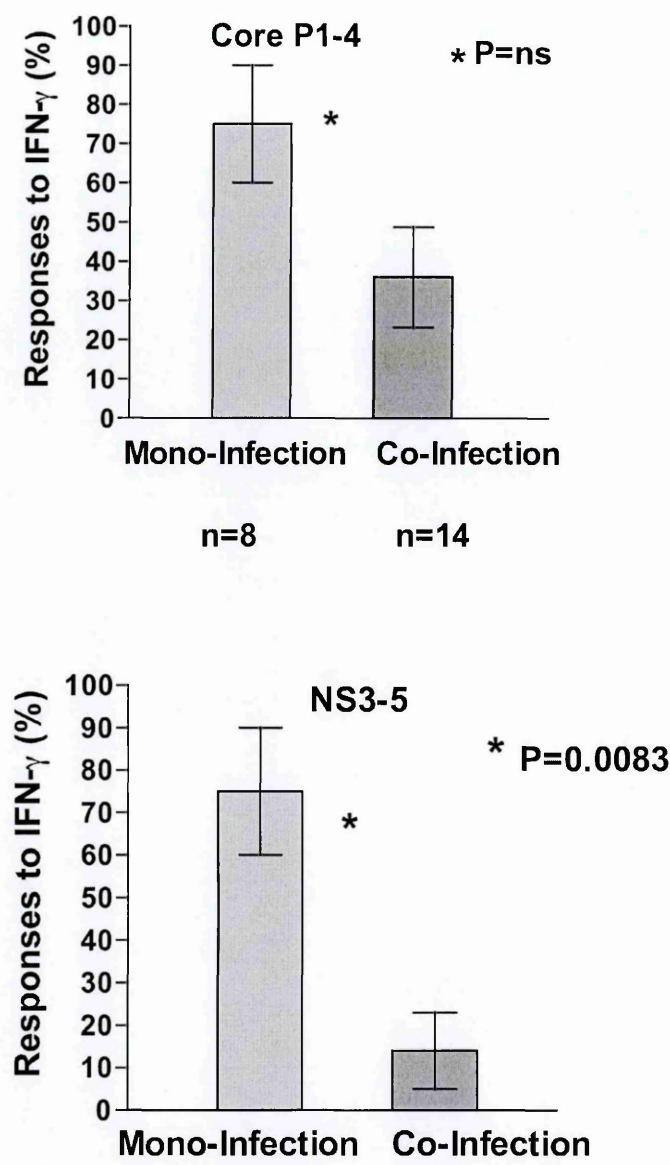
Recently, my colleague Gillian Harcourt et al. analysed HCV-specific CD4<sup>+</sup> T cell responses in a cohort of chronic HCV mono- and HIV/chronic HCV co-infected children with haemophilia. Analysis revealed a major decrease in HCV-specific CD4<sup>+</sup> T cell responses among those who were co-infected, suggesting a cellular mechanism for the loss of control of HCV in co-infected individuals.

In order to investigate whether this cellular mechanism is readily established in the early phase of HCV infection, I had the unique chance to analyse HCV-specific CD4<sup>+</sup> T cell responses to HCV peptides and proteins by *ex vivo* IFN- $\gamma$  ELISpot and CFSE in a cohort of acutely infected HCV individuals with and without HIV co-infection. For both groups T cells were analysed from frozen PBMC.

The co-infected cohort represents a London outbreak of acute HCV in HIV+ men recruited by Mark Danta and colleagues from the Royal Free Hospital London, whereas the mono-infected cohort comprises individuals with acute HCV recruited by Paolo Fabris from the S. Bortolo hospital in Vicenza/Italy. Table 7.1 gives some details about the two groups.

<u>Co-Infection</u> 14/50 cases of acute HCV tested Median Age: 35.7 years Median Length of HIV infection: 4.5 years Median Nadir CD4: 252 cells/ $\mu$ l Median CD4 at Dx: <u>510 cells/<math>\mu</math>l</u> % on HAART: 61% Median HIV VL in those not on HAART: 43 638 Median Peak <u>ALT</u> : 400 U/ml Median <u>HCV VL</u> <u>950 000</u> IU/ml <b>Spontaneous clearance rate: 10%</b>	<u>Mono-Infection</u> 8 cases of acute HCV tested  Median age: 44 years  Median ALT: 1200 U/L  Median HCV VL: 170 000 IU/ml  <b>Spontaneous clearance rate: 50%</b>
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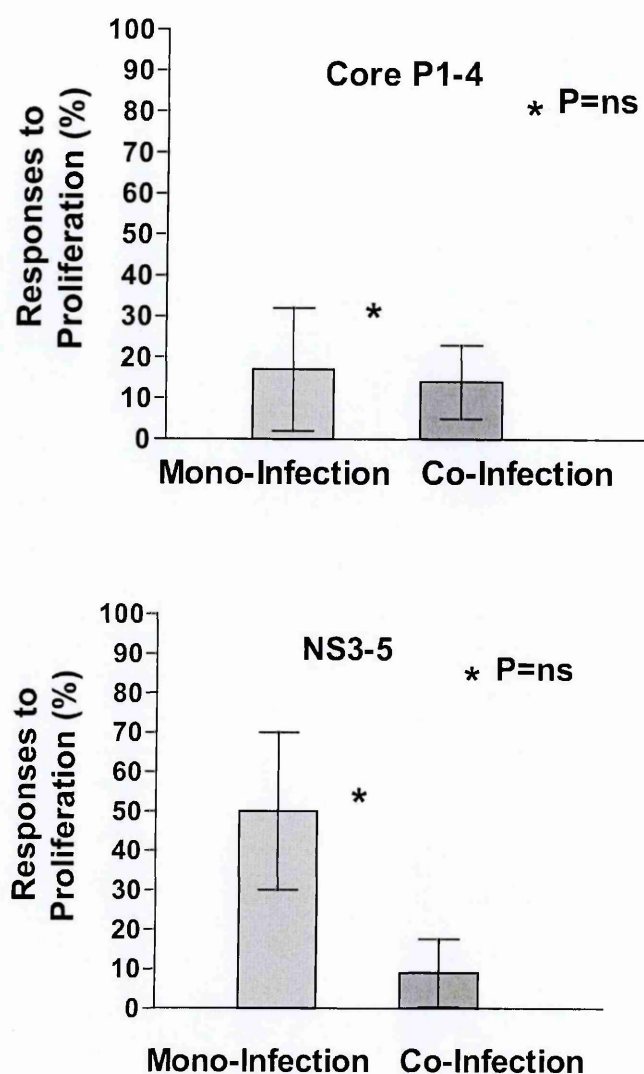
Interestingly, responses could be detected in both groups. However, HCV-specific CD4<sup>+</sup> T cell responses were significantly lower in the co-infected group shown as a lower response to NS3-5 in the IFN- $\gamma$  ELISpot (Fig. 7.1A;  $P=0.0083$ ; Fisher's exact test). In the CFSE assays, although not significant, the percentage of individuals with CD4<sup>+</sup> proliferative responses to NS3-5 was also higher in the mono-infected than in the co-infected group (Fig. 7.1B+ 7.1C;  $P=ns$ ; Fisher's exact test), suggesting early defects in HCV-specific CD4<sup>+</sup> responses in the presence of HIV. Fig. 7.2 shows an example of immune responses for one mono- and one co-infected individual, tested at different time points during the first three months of acute phase HCV infection.



**Fig. 7.1.A. HCV-specific IFN- $\gamma$  T cell responses of mono- and co-infection in the ELISpot.**

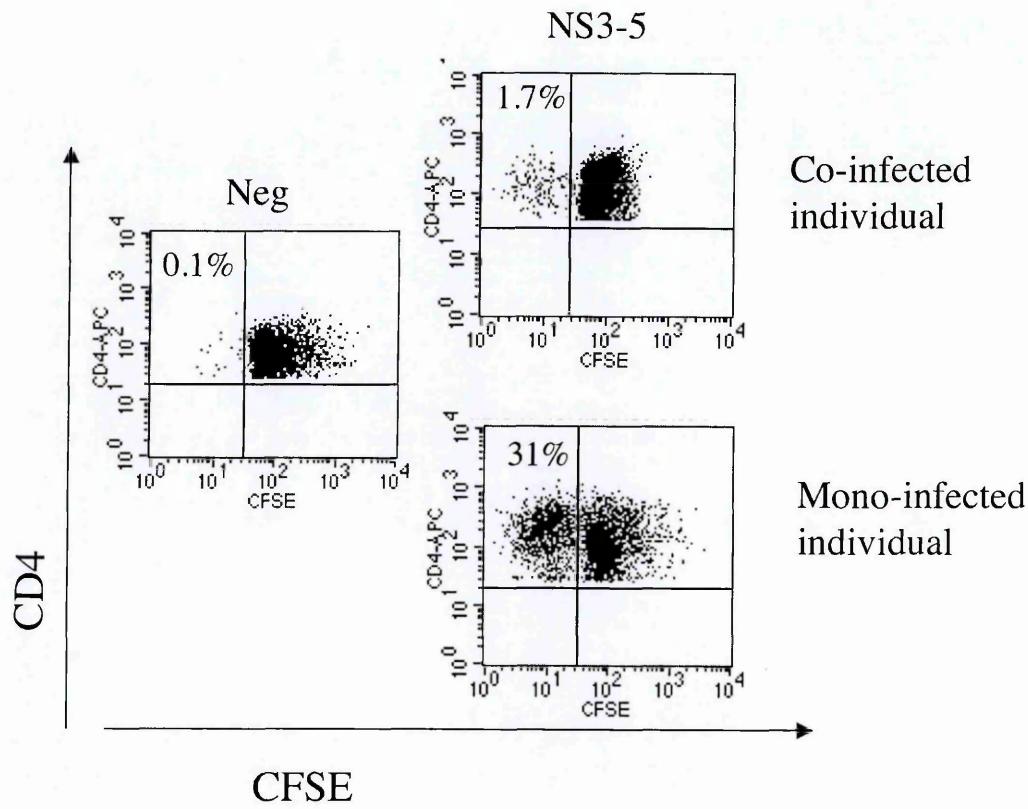
Upper panel: percentage of individuals who had responses to core P1-4. Lower panel: percentage of individuals who had responses to NS3-5. *Results are from the first available time point after diagnosis (first PCR test for HCV RNA). The exact date of infection is not known.*



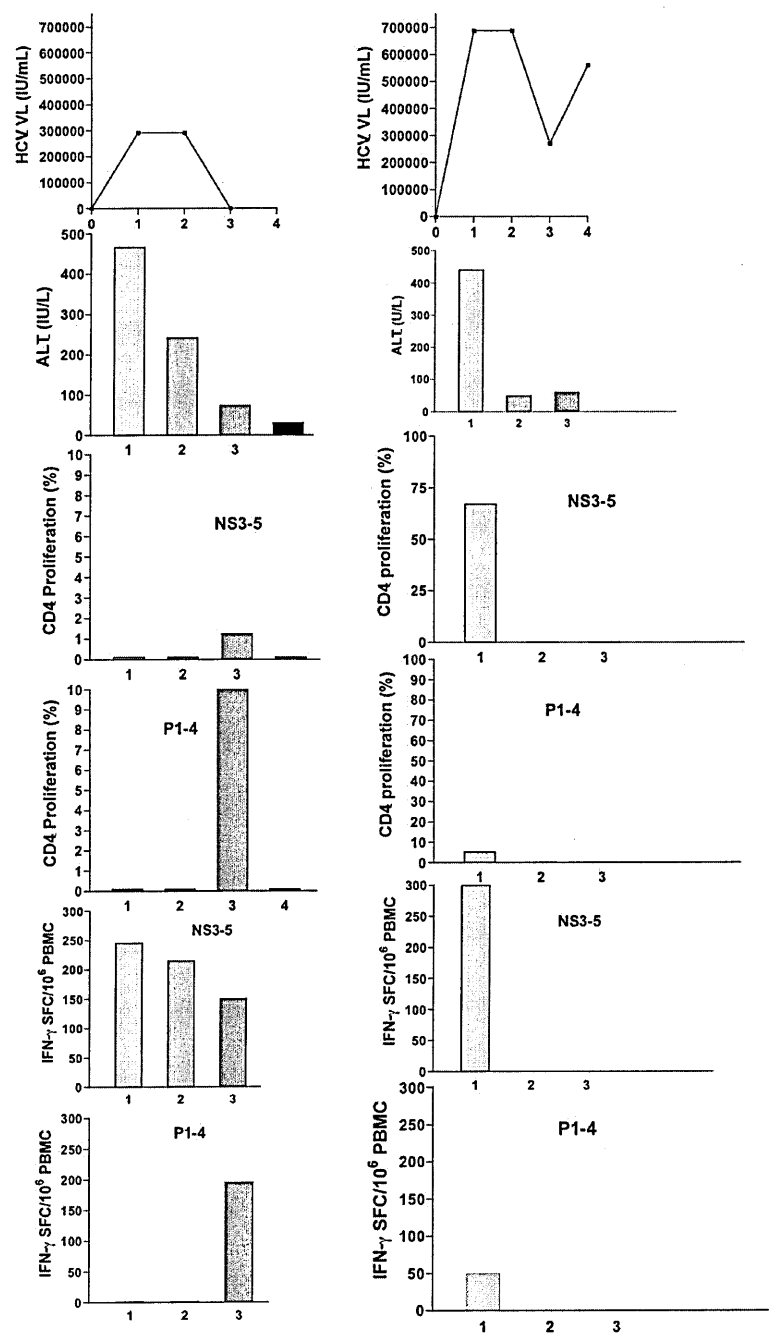


**Fig. 7.1.B. HCV-specific proliferative T cell responses of mono- and co-infection in the CFSE assay.**

Upper panel: percentage of individuals who had responses to core P1-4. Lower panel: percentage of individuals who had responses to NS3-5.



**Fig. 7.1.C. CD4<sup>+</sup> T cell proliferative responses detected in the CFSE assay.**  
Shown for one mono- and one co-infected individual tested for NS3-5.



**Fig. 7.2. Cellular immune responses during acute HCV infection in mono- versus co-infection.**

Examples are shown for one mono-infected (left column) and one co-infected (right column) individual at different timepoints during the first 3 months of acute HCV infection. The mono-infected individual cleared the virus after infection, whereas the

co-infected individual became persistently infected. IFN- $\gamma$  cytokine production and CD4 proliferation are shown for both core P1-4 and the NS3-5, respectively.

These data suggest a significant impact of co-infection on the clinical course and immuno-reactivity during HCV infection is significant. A substantial loss of HCV-specific T cell responses in the presence of HIV has already been shown in other studies. But unlike in our acute cohort, analysis was on patients who were chronically infected with HCV (Lauer et al., 2002) (Kim et al., 2005). It would have been interesting to see whether the reduced CD4<sup>+</sup> T cell responses are a specific characteristic of HCV infection by testing for other antigens such as CMV or EBV. Unfortunately, the low viability of the frozen PBMC in the two cohorts did not allow testing for more antigens. However, other studies have shown that the frequency of IFN- $\gamma$ -producing CMV-specific CD4<sup>+</sup> T cells detectable in a group of HIV-infected individuals was higher than in healthy HIV- negative individuals and was unrelated to the clinical stage of HIV infection (Waldrop et al., 1997), providing further evidence of the sensitivity of the HCV-specific CD4<sup>+</sup> T cell responses to the suppressive effect of HIV.

Although about 60% of our co-infected patients were on HAART, a restoration of the CD4 count and delay of progression to AIDS did not seem to have an effect on preservation of HCV-specific CD4<sup>+</sup> T cells.

That HAART does not seem to have a clear positive effect on HCV outcome has been reported elsewhere (Kottlil et al., 2004). Further studies are required to identify the mechanisms behind the failure of HCV-specific T cell responses in the presence of HIV.

These findings emphasise the potential importance of HCV-specific CD4<sup>+</sup> T cell responses in control of chronic infection. Further analysis in particular should include whether HAART influences HCV-specific CD4<sup>+</sup> T cell responses to HCV, how this differs from responses to other antigens, and how this relates to any changes in the HCV viral load observed. Finally, the sensitivity of HCV-specific CD4<sup>+</sup> T cells to HIV may relate to features of their site, phenotype, activation status or turnover; these issues are discussed elsewhere in this thesis. HIV/HCV co-infection represents a major clinical issue and one where simple immunological data in well defined cohorts could provide important insights in future.

## **8. Concluding remarks**

The work in the project has furthered our understanding of CD4<sup>+</sup> T cell responses to HCV in a variety of clinical settings. Firstly, I demonstrated persistence of such responses in the absence of a classical antibody response in the RIBA indeterminate cohort. This finding was revisited at the end of the thesis in the antibody-deficient group, where T cell responses appear to be protective and are maintained in the absence of antibody.

In persistent infection I have begun to define the quantity and quality of CD4<sup>+</sup> T cell responses – an area, which had previously been addressed largely using only culture techniques. More work is required to further identify the targets of the response in persistent infection, the quality of the responses and their role *in vivo*. It does appear – given the major clinical impact of HIV on HCV, as discussed in this chapter, that disturbance of the HCV-specific CD4<sup>+</sup> T cell responses in chronic HCV infection does have a big effect on long term outcome. Having established the importance of these subsets and to a certain extent the tools to study them, I hope to be able to explore these better in future studies of HCV.

## List of publications

Semmo N, Krashias G, Willberg C, Klenerman P:

**Sustained populations of “effector memory” CD4<sup>+</sup> T cells in persistent hepatitis C virus infection.** J Gen Virol (submitted)

Klenerman P and Semmo N:

**Cellular immune responses against persistent hepatitis C virus: gone but not forgotten.** Gut 2006;55:914-916

Semmo N, Lucas M, Krashias G, Lauer G, Chapel H, Klenerman P: **Maintenance of HCV-specific T cell responses in antibody-deficient patients a decade after early therapy.** BLOOD 2006;55:914-916

Semmo N, Day CL, Ward SM, Lucas M, Harcourt G, Loughry A, Klenerman P: **Preferential loss of IL-2 secreting CD4<sup>+</sup> T helper cells in chronic HCV infection.** HEPATOLOGY 2005;41(5):1019-1028

Semmo N, Harcourt G, Klenerman P, Smith N:

**HCV-indeterminate blood donors or occult HCV infection? Reply.**

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